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Objective: Human basophils and mast cells have βI integrin receptors that modulate IgE-mediated release and in the case of asthmatic donors these βI integrin receptors directly induce release. Our goal is to determine the signal transduction mechanisms that mediate these two effects. We will determine what signal transduction pathways occur during integrin receptor activation as well as determining where the IgE-mediated and integrin receptor pathways interact to produce the effects noted above.

Approach: The primary cell model for these studies will be the human basophil. We will use technologies we have developed to characterize IgE-mediated signal transduction in basophils. These studies will initially focus on events that occur immediately after stimulation.

Accomplishments (throughout award period): The goals of the proposed studies were founded on the basic observation that some integrins could modulate IgE-mediated release from human basophils. At the beginning of the funding period, no studies of the early signaling events which followed stimulation through the high affinity IgE receptor had been made in either human basophils or mast cells. It was felt that to examine integrin signaling properly, it was necessary to first lay the groundwork for what signaling events could be measured in human basophils. As noted in previous reports, these cells are notoriously difficult to study. It has taken 3 years to feel relatively comfortable with nature of early signaling in human basophils, although it has many elements similar to IgE-mediated signaling in other mast cell models like the RBL-2H3 cell.

Our efforts began with a general exploration of the number and type of proteins that are phosphorylated on tyrosines early in the signaling reaction. Tyrosine phosphorylation appears to be involved in most of the early well characterized signaling components studied in RBL-2H3 cells as well as other models of immunoreceptor activation. Using whole cell lysates analyzed by Western blotting with anti-phosphotyrosine antibodies, a large number of proteins were observed to have kinetic time courses consistent with components of secretion whose activation preceded mediator release in basophils, thus identifying them as signaling molecules that operate early during stimulation. As expected, two early participants were found to be lyn and syk kinase, two members of tyrosine kinase families known to participate in all known immunoreceptor signaling. The purpose of using whole cell lysates was to get a sense of the variety or proteins that were tyrosine phosphorylated and while some of the proteins have now been identified, others remain unidentified. The

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13. ABSTRACT *(Maximum 200 words)*

Critical progress has been made in the identification and characterization of cells and mediators involved in allergic inflammation. Accumulating evidence supports the importance of cell adhesion molecule expression as an initiating process in tissue inflammation. Despite progress made to date, much is still unknown about the exact mechanisms responsible for this inflammatory response. Scientists have been working to understand the selective cell recruitment operating in allergic disease with the hope of discovering therapeutic intervention strategies that will prevent the accumulation of unwanted cells in inflamed airways. Research has been directed at developing various approaches to generate specific antagonists. Some approaches under study interupt airway inflammation in its early stages during leukocyte-endothelial interactions. Other approaches inhibit cell recruitment at the endothelial wall. Many studies have been done, both in vivo and in vitro, and the advances that have been made suggest that these therapeutic interventions may be the keys to controlling and, possibly, curing asthma and allergic reactions.

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goal was to understand down-regulation, so we focused our efforts on the earliest known activities in this reaction, the activity of lyn and syk kinase. The first year of study helped to exclude some proteins simply because they were found to be phosphorylated during stimulation with a wide variety of secretagogues. For example, pyk2 is prominently phosphorylated during stimulation with every secretagogue known. As expected, phosphorylation of lyn and syk kinase did not occur with non-IgE mediated stimuli. The second year of study confirmed the basic observations of lyn and syk kinase phosphorylation using the more selective technique of immunoprecipitation. This technique also required larger numbers of basophils for each experiment and work proceeded slowly.

We began to re-examine integrin modulation of IgE-mediated secretion but found that available antibodies which aggregated cell surface integrins had only modest inhibitory effects on IgE-mediated histamine release from basophils. We reasoned that if we were going to explore how aggregation of integrins down-regulated IgE-mediated release, we should first examine the limitations of the methodologies that we were using to examine early signaling. In addition to the necessary characterization of early kinase signaling, especially that of syk kinase, we used a model of a down-regulatory reaction that we knew would reduce the cellular response to zero -- the down-regulation that naturally occurs through the IgE receptor itself. These studies have consumed the efforts of the last year and while a great deal has been learned, we have only recently laid sufficient groundwork that we may examine how integrin signaling may modify these early events, if at all. These studies will be continued outside the funding period.

The first surprising observation was that phosphorylation of syk kinase (which can be viewed as a partial indicator of its activity) is prolonged during stimulation with polyclonal anti-IgE antibody while it is transient when antigens are used. The important caveat to the antigen study is that the cells were first sensitized with an antigen-specific IgE antibody. This procedure is limited in that only unoccupied IgE receptors can be sensitized by the antigen-specific IgE of choice. Since normal circulating basophils express many receptors but only a modest number (<5000) of unoccupied receptors, sensitization results in a relatively low density of antigen-specific IgE. Some background is necessary to understand the relevance of this information. Previous studies established that two forms of down-regulation occur during IgE-mediated secretion, one which is specific to the antigen being used and one which effects the subsequent secretory response to other non-crossreacting antigens. The later form of down-regulation has been termed nonspecific desensitization, which is somewhat of a misnomer in that the "nonspecificity" applies only to antigens operating through the FceRI. In other words, for cell preparations that have been treated to induce complete nonspecific desensitization, secretion can be induced through other non-FceRI dependent receptors. The amount of nonspecific desensitization experienced by a cell is a function of the density of cell surface IgE for the antigen used to desensitize the cell. For example, if a cell has a cell surface density of ragweed-specific IgE of 5000 molecules and a cell surface density of 5000 molecules of rye grass-specific IgE, desensitizing the cell with ragweed antigen(s) will have little effect on rye grass antigen-induced release. However, if there are 25,000 molecules of ragweed-specific IgE per basophil (and 5,000 rye grass-specific IgE molecules per cell) and the cell is desensitized with ragweed antigen(s), there will be little response to stimulation with rye grass antigens, i.e., full

nonspecific desensitization. The theoretical construct for these observations has for some time been that specific desensitization results from modification of a very early receptor-associated step in signaling while nonspecific desensitization alters some shared element further down-stream the signaling cascade. The implication of the syk phosphorylation kinetics data is that specific desensitization results in modifications of events prior to syk phosphorylation while nonspecific desensitization results in modifications that follow syk phosphorylation. The low level of sensitization with antigen-specific IgE would normally be associated with conditions that lead to specific desensitization, so the observation that syk phosphorylation is transient indicates that whatever process leads to specific desensitization, it probably operates on events preceding syk phosphorylation. In contrast, when the cells are stimulated with polyclonal anti-IgE antibody, previous studies note that crosslinking the entire complement of cell surface IgE leads to complete nonspecific desensitization (and not just for the trivial reason that all cell surface IgE has been crosslinked). The observation that syk phosphorylation is persistent within this time frame (even though histamine release has stopped), suggests that nonspecific desensitization alters events down-stream of syk phosphorylation or results in alterations to syk itself. In related studies we found a region in the signaling cascade that is effected by nonspecific desensitization. Shc is an adapter protein that is phosphorylated by syk kinase in other cell models. Like syk phosphorylation, Shc phosphorylation is also persistent. By a sequence of steps not yet completely clear for human basophils, p21ras is activated downstream of syk and Shc phosphorylation. However, in basophils stimulated with anti-IgE antibody, p21ras activation is transient, as are signaling kinases that lie downstream of p21ras activation. Therefore, nonspecific desensitization may operate on those elements of the cascade that lie between Shc phosphorylation and p21ras activation, or on p21ras itself.

These results have also been found for desensitized cells. For the experiments described in the preceding paragraph, the analysis was based on observations of events occurring during active secretion. Desensitization experiments are an artificial method of revealing the down-regulatory steps that may occur during active secretion. If calcium is excluded from the reaction buffers, stimulation through FceRI continues to initiate some signaling but without ongoing secretion (because secretion requires extracellular calcium). When calcium is added back to the buffers, secretion begins but has been blunted by the preceding period of down-regulation. If the desensitization phase of the reaction is extended to 60-120 minutes, there is no secretion when calcium is returned to the buffers. When basophils were sensitized with antigen-specific IgE and desensitized with antigen for 60 minutes (in the absence of extracellular calcium), the subsequent addition of calcium did not lead to any syk phosphorylation (which was transiently phosphorylated during the incubation without extracellular calcium). In contrast, desensitizing the cells with anti-IgE antibody had little influence on the immediate syk phosphorylation that followed addition of calcium. This behavior was similar to the kinetic behavior during active secretion. Although anti-IgE desensitization markedly suppressed histamine release, syk phosphorylation continued. There were two potentially important observations about syk phosphorylation in cells desensitized with anti-IgE antibody. The first was that although syk phosphorylation was similar in control cells (not desensitized) and desensitized cells five and ten minutes following the addition of calcium, by 30 minutes, syk phosphorylation was decreased in the desensitized cells compared to non-desensitized cells. The second observation was that

immunoprecipitation of syk was different in desensitized cells. Not as much syk could be adsorbed to the affinity matrix when the cells lysates were derived from desensitized cells. These two observations suggest that there may be a modification to syk itself in cells desensitized with anti-IgE antibody. The nature of this modification is under study.

These studies have set some guidelines for future experiments on regulation of IgE-mediated secretion. In particular, syk phosphorylation provides a useful indicator of two kinds of down-regulation.

Significance: Basophils are an integral component of the allergic reaction. The factors which regulate their function, in particular, their secretion of inflammatory mediators, are only crudely understood. Furthermore, for the regulatory factors that are known, very little is known about the underlying mechanisms of their action. The studies supported by this grant have been instrumental is establishing some of the basic characteristics for early IgE-mediated signaling in a *human* cell believed to be important in allergic reactions. This work provides the basis for examining regulators of secretion, like adhesion molecules.

Publications, Reports and abstracts:

Abstracts:

Lavens-Phillips, S., and MacGlashan, D. W., Jr. (1998) The role of p53/56 lyn and p72syk in IgE-mediated signal transduction in human basophils. *FASEB J* **12,** 591a.

MacGlashan, D.W., Jr., J. McKenzie, S.E. Lavens-Phillips, A.J. Henry, B.J. Sutton, H.J. Gould. 1998. IgE interacts with FcεRI to induce up-regulation on human basophils. *FASEB J* 12:A6137.

Lavens-Phillips, S. E., and MacGlashan, D. W., Jr. (1999) Pharmacology of human basophil desensitization. *Faseb J.* **13,** A338. (note that this poster was presented with data not available at the time the abstract was composed for submission and included much of the data presented in the later part of the narrative)

Publications:

D. W. MacGlashan, Jr. S. Laven-Phillips & K. Miura. IgE-mediated desensitization in human basophils and mast cells. Frontiers in Bioscience (1998) webpage: http://www.bioscience.org/1998/v3/d/macglash/list.htm.

Lavens-Phillips, S., K. Miura, J.T. Schroeder and D.W. MacGlashan, Jr. The effect of PP1 and PP2 on IgE-mediated signaling events in human basophils. submitted for publication to Biochem. Pharm.

Lavens-Phillips, S., K. Miura, and D.W. MacGlashan, Jr. Changes in the phosphorylation of syk kinase differentiates IgE-mediated specific and nonspecific desensitization in human basophils. submitted for publication to J. Immunol.

Lavens-Phillips, S., K. Miura, and D.W. MacGlashan, Jr. Picatannol does not inhibit secretion from human basophils through inhibition of syk kinase. manuscript in preparation.

MacGlashan, Jr., D.W. and S. Lavens-Phillips Pharmacology of the early calcium response and its relationship to non-releasing basophils. manuscript in preparation.

Changes in the Phosphorylation of Syk Kinase Differentiates IgE-Mediated Specific and Nonspecific Desensitization in Human Basophils

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Abstract

IgE-mediated down-regulation of secretion from basophils and mast cells is an important component of the overall cellular response which determines the ultimate extent of mediator release. The down-regulatory process that occurs during active secretion has also been associated with the methodological phenomenon called desensitization. The mechanisms underlying desensitization are not understood. Human basophils were examined for changes in syk phosphorylation under conditions associated with either specific or nonspecific desensitization. Conditions associated with specific desensitization resulted in transient syk phosphorylation in the presence or absence of extracellular calcium while conditions associated with nonspecific desensitization resulted in sustained syk phosphorylation. Specific desensitization ablated syk phosphorylation while nonspecific desensitization did not. Treatment of the cells with the lyn kinase inhibitor, PP1, reversed either form of desensitization while inhibitors of PKC had no effect. The data suggest that specific desensitization results from alterations in signaling that precede syk phosphorylation while nonspecific desensitization results from changes to syk itself or more likely from alterations to elements downstream of syk kinase. Both forms of desensitization appear to require the activity of lyn kinase.

Introduction

IgE-mediated secretion of mediators from human basophils results from a balance of reactions which both drive secretion or serve to limit its extent. For human basophils, the reactions that serve to limit secretion have generally been associated with the experimental phenomenon known as desensitization. This phenomenon is operationally demonstrated by stimulating basophils with antigen under conditions suboptimal for secretion, usually the absence of extracellular calcium (1). The longer this is done, the more poorly the cells secrete when calcium is returned to the medium. The decay in responsiveness over time has a superficial resemblance to first order decay kinetics. When desensitized, basophils cease secreting all 3 major classes of mediators (granule contents such as histamine, rapidly formed lipid mediator such as LTC4 and slowly formed mediators such as IL-4). Desensitization with one antigen may affect secretion induced by another non-crossreacting antigen (in cells displaying IgE with more than one antigenic specificity) but the degree to which other antigens are affected depends on the density of IgE specific for the antigen used to desensitize the cell. Thus, desensitization may be specific or nonspecific in nature relative to all the IgE specificities displayed on the cell surface. However, while desensitization induced through the IgE receptor may affect other IgE receptor mediated signaling, it has no inhibitory effect on signaling induced by a variety of other secretagogues. This set of observations suggests that specific pathways relevant only to IgE-mediated release are altered during desensitization. It also appears that desensitization alters internal signaling since expression of the receptor (and its associated IgE antibody) is not altered in desensitized human basophils. The nature of this alteration in signaling remains unknown.

The earliest known events that follow aggregation of FceRI involve the phosphorylation and activation of two receptor associated tyrosine kinases. The src-family kinase, lyn, has been implicated in the initial phosphorylation of the FceRI b and g subunits which in turn leads to the

recruitment of syk kinase to the ITAMs of the g2 subunits. Phosphorylation of syk ensues and is generally considered a marker of the activation of syk (although there are inhibitory phosphorylation as well on syk). To further delineate the alterations in signaling that follow desensitization, we have examined the changes in syk phosphorylation occurring in non-desensitized cells as compared to desensitized cells. A more complete examination of the self-regulating process we have termed desensitization requires three types of tests. Since desensitization is presumably an integral part of normal secretory response, the characteristics of the kinetics during active secretion should be a measure of the self-regulatory process in the whole reaction. Since desensitization is observed by first treating cells with antigen in the absence of extracellular calcium, the kinetic characteristics of the event being examined (syk phosphorylation in this case) is also useful information. For example, the event may not happen at all without extracellular calcium. Finally, following a desensitization phase, an examination of the occurrence of the event in desensitized vs. non-desensitized cells is also useful. We have examined the changes in syk phosphorylation under each of these circumstances and also compared the characteristics of the response when the cells were experiencing either specific or nonspecific desensitization.

Materials and Methods

Materials: Formyl methionyl-leucyl-phenylalanine (fMLP), Phorbol 12-myristate 13-acetate (PMA), human IgG, piperazine N, N' bis 2 ethane sulphonic acid (PIPES), glucose, ethyleneglycol-bis-N,N,N',N'-tetraacetic acid, (EGTA), ethylenediaminetetraacetic acid (EDTA), fetal calf serum, bovine serum albumin (BSA), human serum albumin (HSA), perchloric acid, sodium orthovanadate, benzamidine, aprotinin, phenylmethylsulphonyl fluoride (PMSF), sodium flouride, 2-mercaptoethanol and nonidet P-40 were all purchased from Sigma Chemical Co. (MO, USA). RPMI 1640 supplemented with 25mM hepes and L-glutamine was bought from Biowhittaker (MO, USA) while gentamycin was obtained from Gibco BRL (NY, USA). Sodium dodecylsulphate (SDS) and tris were purchased from Bio-Rad (NY, USA). Protein G sepharose

and Percoll were purchased from Pharmacia Biotec (NJ, USA). 4-20% tris-glycine gels and 2x sample buffer were bought from Novex (CA, USA) while biotinylated molecular weight markers were purchased from New England Biolabs (MA, USA). The antibody cocktail and columns used in the negative selection of human basophils were purchased from Miltenyi Biotech (CA, USA). Mouse anti-human p72syk was purchased from Santa Cruz Biotechnology Inc. (CA, USA), while the mouse anti-phosphotyrosine monoclonal antibody, 4G10 was purchased from Upstate Biotechnology (NY, USA). Sheep anti-mouse Ig horseradish peroxidase, donkey anti-rabbit Ig horseradish peroxidase, streptavidin horseradish peroxidase conjugate, ECL western blotting detection agents and ECL hyperfilm were all purchased from Amersham (IL, USA). Ionomycin, Bis-indolylmaleimide I and II, Ro 31-8820, calphostin C, staurosporine and Go-6976 were all purchased from Calciochem (La Jolla, Ca). PP1 and PP2 were purchased from Biomol (PA, USA). Goat anti-human IgE was prepared as described previously; the antibody used for these studies represented the IgG fraction of goat serum prepared by DE-52 chromatography (2). benzylpenicilloyl (BPO)-HSA and BPO-EACA (e-aminocaproic acid) were synthesized as previously described (3). GP-120(HIV)-OVA (ovalbumin) conjugate was the gift of Dr Francis davis of Tanox, Corp (Houston, TX). All other reagents used were of the highest grade available.

Buffers: PIPES buffer contained 25 mM PIPES, 110 mM NaCl and 5 mM KCl adjusted to pH 7.4 with 1 N HCl; PIPES-albumin-glucose (PAG) also contained 0.003% (w/v) human serum albumin (HSA) and 0.1% (w/v) glucose: PAGCM was supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$; elutriation buffer for the purification of human basophils contained 10% PIPES, 0.1% (w/v) glucose and 0.25% (w/v) bovine serum albumin (BSA). Lysis buffer contained 20 mM Tris (pH 7.8), 150 mM sodium chloride, 1% nonidet P-40, 5% glycerol, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM benzamidine, 1 µg/ml aprotinin. In the electrophoresis studies, 2x Sample buffer contained 0.5 M Tris-HCl, pH 6.8, 10% (w/v) SDS, 0.1% bromophenol blue, 20% glycerol, and 5% mercaptoethanol; TBST buffer contained 12 mM Tris base (pH7.5), 150 mM NaCl and 0.05% tween-20; running buffer

contained 25 mM Tris base, 192 mM glycine and 0.1% SDS; transfer buffer contained 12 mM Tris base, 96 mM glycine and 20% methanol; stripping buffer contained 7M guanidine hydrochloride.

Isolation of human basophils: Basophils were purified from buffy coat cells, obtained from normal donors undergoing leukapheresis as previously desribed (4). The leukocytes were partially purified by Percoll density gradient and counter current elutriation. The basophils are then placed in to culture (RPMI 1640 with 2% FCS and 20 mg/ml gentamycin) for one hour after elutriation then further purified on a subsequent two step Percoll gradient (1.066/1.079). After culturing overnight in RPMI 1640 supplemented with 2% FCS, gentamycin and 30 pM IL-3 (a concentration predetermined to only maintain better viability but with no measurable priming effect on basophil function), the cells were further purified by negative selection. For these studies, basophil purities were >90%.

Cell counting: Mast cells and basophils were stained with Alcian blue (5) and counted in a Spiers Levy hemocytometer.

Histamine release and Sensitization: For the pharmacological and desensitization experiments, basophils were obtained by venipuncture and partially enriched over a single step Percoll gradient. The cells were then challenged and supernatants were harvested for analysis by automated fluorimetry (6). Histamine release is expressed as a ratio of sample to total histamine, obtained by lysis of an equivalent number of cells with perchloric acid, after subtracting spontaneous release (range 1% to 5%). For the desensitization experiments using specific antigens, the basophils were first sensitized with a mixture of two specific IgE antibodies, anti-penicillin (BPO) at a concentration of 2 µg/ml and anti-GP120 at 2 µg/ml for 30 minutes at 37° C in RPMI buffer containing 0.1 mM BPO-EACA, 10 µg/ml heparin and 1 mM EDTA. After washing, the cells were used for the experiments described. For the data analysis in these experiments, the relevant analysis within each experiment required expressing the amount of desensitization as a fraction of the control histamine release (non-desensitized cells). After expressing the data this way, the results were averaged for the replicate experiments and the data was converted back to absolute histamine release by multiplying the averaged ratios by the average control histamine release. With

this approach, control histamine release initially had a value of 1.0 (as other data is expressed as a fraction of 1.0) times the actual average release, therefore there are no standard error bars in figures ? and ? for these control values.

Cell stimulation and preparation of cell lysate: Purified human basophils were resuspended in PAGCM prior to their stimulation with either 0.2 μg/ml anti-IgE. 100 μl aliquots were then removed at various time points and added to 900 μl of ice-cold PAGCM. The cell free supernatants were then removed for histamine analysis and the cell pellet lysed by the addition of 40 μl of 1x SDS sample buffer/10^6 cells. In some experiments, human basophils were desensitized by stimulating them with anti-IgE (0.2 μg/ml) in the absence of extracellular calcium. For these studies the cells were washed twice, then resuspended in either PAG (a calcium free buffer) or PAGCM (a calcium containing buffer) for 5 minutes followed by the addition of 2.5mM EGTA to remove the extracellular calcium. In some experiments, calcium was added back to the medium after stimulation to confirm that the basophils had desensitized, i.e. no further histamine release or increase in tyrosine phosphorylation was observed. In some experiments, histamine release was measured from basophils which were resuspended in PAG then stimulated with 1μM fMLP to confirm that desensitization was limited to the anti-IgE signaling pathway. In all cases, the phosphotyrosine containing proteins were analyzed as described below.

Immunoprecipitation: Lysates from $2x10^6$ basophils per condition were precleared with protein G sepharose beads for 30 minutes at 4° C. The precleared lysates were then washed three times in ice-cold lysis buffer and incubated with 1 μg/ml anti-p72syk prebound to protein G sepharose beads. After gentle rotation for 1 hour at 4° C, the beads were washed four times in ice-cold lysis buffer. The immunoprecipitated proteins were then eluted from the beads by boiling in 1x SDS sample buffer. Control experiments revealed that an irrelevant IgG antibody or mouse anti-human p72syk in the absence of lysate did not pull down syk in the immunoprecipitates.

Blotting of proteins: Proteins were separated in a 4-20% tris-glycine gel under reducing conditions and electrotransferred on to a nitrocellulose membrane. The free binding sites were blocked by incubating the membrane overnight at 4° C with 3% BSA in TBST. The nitrocellulose membranes

were then incubated with 0.5 μg/ml of the anti-phosphotyrosine monoclonal antibody, 4G10, in 1% BSA/TBST for 1 hour at room temperature. The membrane was then thoroughly washed with TBST prior to the addition of an anti-mouse horseradish peroxidase conjugate (1:3000 dilution) for 1 hour at room temperature. After further extensive washing of the membranes with TBST the phosphoproteins were visualized using Enhanced Chemiluminescence. The nitrocellulose membrane was exposed to ECL hyperfilm for 15 seconds to 10 minutes. In some experiments, the nitrocellulose membrane was stripped for 30 minutes at room temperature then reprobed with 0.2 μg/ml mouse anti-human p72syk. After exposure to chemiluminescence detection agents, the intensity of each band was determined using densitometric analysis. In some cases, the increase in tyrosine phosphorylation of a relevant band was then determined as a fraction of the maximum increase found during the time course.

Results

In accord with recent studies of human basophilic leukemia cells or normal peripheral blood basophils, we have also recently demonstrated that normal peripheral blood basophils show early tyrosine phosphorylation of both lyn and syk following IgE-mediated stimulation (7). While not the earliest tyrosine kinase involved in signaling, syk is thought to be the point from which many different pathways diverge to generate an array of functional responses in basophils and mast cells. We have therefore examined the characteristics of this central signaling component from the three perspectives discussed above. For these studies, syk phosphorylation was taken as a marker of its activation although this point will receive further discussion below.

Kinetics of syk phosphorylation during stimulation ±extracellular calcium

Purified human basophils (n=3) were stimulated with an optimal concentration of anti-IgE antibody for up to 2 hours (figure 1A). These long term kinetic experiments indicate that syk phosphorylation remained elevated for at least 2 hours following anti-IgE stimulation. Desensitization is operationally demonstrated by prior stimulation in the absence of extracellular

calcium. As demonstrated in figure 1C, experiments examining the kinetics of the response in the absence of extracellular calcium indicated that phosphorylation was also maintained for at least one hour at levels similar to the 5 minute point time point, as found for stimulation in the presence of extracellular calcium. We noted, however, that syk phosphorylation was less in cells challenged in the absence of extracellular calcium. For experiments where stimulation in the presence and absence of extracellular calcium were examined in he same cells, the average ratio of syk phosphorylation in the absence versus presence of extracellular calcium was 0.6 (n=7), although there was variability among preparations (histamine release was 38±9% in the presence of extracellular calcium and 6±4% in its absence). These results are shown in figure 1B. Similar blunting was noted when stimulated cells were examined at earlier time points (data not shown).

Anti-IgE antibody causes nonspecific desensitization[2], so we next examined conditions normally associated with specific desensitization, stimulation of sensitized basophils with antigen. Basophils were sensitized with penicillin (BPO)-specific IgE, maximally loading the few available unoccupied receptors typical in these preparations (see methods). The cells were then stimulated with an optimal concentration of a relatively low valency BPO-human serum albumin conjugate (BPO(11)-HSA) under conditions similar to those used for anti-IgE antibody. Figure 2A and 2C show the kinetic time course in the presence an absence of extracellular calcium. Unlike anti-IgE antibody, stimulation with antigen caused a transient phosphorylation of syk kinase. There was consistent and modest difference in the rate of return to resting levels in cells stimulated in the presence or absence of extracellular calcium but in both cases, phosphorylation was near resting levels by two hours. As with anti-IgE antibody, there was a blunting of syk phosphorylation when cells were stimulated in the absence of extracellular calcium. Figure 2B shows that this ratio, in cells were both conditions were employed, was similar to that found for anti-IgE antibody.

Sensitizing the cells with BPO-specific IgE provided the opportunity to test whether the residual syk phosphorylation at later times remained dependent on the presence of aggregates. Figures 2A

and 2C demonstrate that the addition of a monovalent penicillin, BPO-EACA (0.1 mM), at 5 minutes and 60 minutes, caused a rapid loss of syk phosphorylation. It should be noted that in two of these experiments, the response to anti-IgE antibody was also being studied. Curiously, while the relative syk phosphorylation response to BPO-HSA was 0.30±0.10 of the response to anti-IgE antibody, histamine release was greater following challenge with BPO-HSA (45% vs. 34%, for BPO-HSA vs. anti-IgE, respectively). These results suggested that two divergent kinetic characteristics followed stimulation that typically resulted in specific or nonspecific desensitization. These results prompted an examination of both types of stimulation in the desensitization experiments discussed next.

Effect of desensitization on the re-challenge response

Previous investigations have demonstrated that when human basophils are desensitized, histamine release, induced by an optimal concentration of anti-IgE, is reduced to zero over a specific time period with a $t_{1/2}$ of approximately 15 minutes (8). We stimulated purified human basophils with anti-IgE (0.2 μg/ml) in the absence of calcium for 60 minutes. At the 60 minute time point buffer was added containing enough calcium and magnesium to result in a final concentration of 1 mM. The phosphorylation of syk was then determined at several time points following the addition of extracellular calcium. For all experiments, phosphorylation after 5 minutes was examined and in a subset of experiments, the response at 10 or 30 minutes was also examined. Syk phosphorylation was measured by densitometry and the ratio of the densities with and without desensitization was calculated and averaged. In some cases the cells were sensitized with BPO-specific IgE, as above, and desensitized with BPO-HSA for 60 minutes prior to the addition of calcium/magnesium. Figure 3 shows some representative Western blots for these experiments. The top panel provides an example of desensitization with BPO-HSA and anti-IgE antibody in cells sensitized with BPO-specific IgE. The measurement of syk phosphorylation was made 5 minutes after the introduction of calcium. The lower two panels show results for cells desensitized with anti-IgE antibody with measurements after the addition of calcium at 5 and 30 minutes. Aside from more obvious results

that are summarized in figure 4, there are two additional features seen in the blots themselves. The first is that the kinetics of syk phosphorylation in non-desensitized cells, i.e., cells which were incubated for 60 minutes in the absence of extracellular calcium and without stimulus, appears slower than we observed when this incubation was not done. Compare the intensity of the 5' and 30' bands in panels B and C. The 5' point is less than 50% of the 30' point while data from figure 1A and B indicate that it should be greater than 50%. The second observation may provide an important clue to the changes in syk during desensitization. Recovery of syk by immunoadsorption is almost always less efficient when the lysates are derived from desensitized cells. On average, the intensity of the syk band after stripping the 4G10 blots and re-probing with anti-syk antibodies, is 70±15% of the non-desensitized cells. We ran two experiments where parallel samples of cells ± desensitization were lysed in sample buffer and Western blots of the whole cell lysates examined for the presence of syk. In both cases, while the IP data indicated a loss of syk, the whole cell lysates indicated no loss. To examine this further, we desensitized some basophils with anti-IgE antibody, lysed the cells and immunoadsorbed with the 4D10/Staph G beads, then split the beads into two samples. One sample was sham treated with calf alkaline phosphatase while the other was incubated with alkaline phosphatase for 1 hour. Sample buffer was then added to the washed beads and Western blotting revealed similar levels of syk. These studies ruled out a transblot problem due to differences in phosphorylation of syk (as was found in studies of Shc).

As figure 4 demonstrates, anti-IgE induced p72syk phosphorylation in immunoprecipitates was not suppressed in desensitized basophils compared with non-desensitized cells. In contrast, antigen (BPO-HSA) induced desensitization of human basophils significantly suppressed syk phosphorylation to near basal levels. Histamine release performed in parallel showed that 60 minutes of desensitization led to release that averaged 16±7% of the non-desensitized response (38±9% release). In four of the seven experiments, we examined the level of syk phosphorylation following 60 minutes of desensitization at 5 and 30 minutes following the addition of extracellular

calcium in order to determine whether the kinetics of the response at longer times was similar in control and desensitized cells. While the five minute measurements indicated little difference between cells ± desensitization, by 30 minutes, syk phosphorylation in desensitized cells was 50% of the response in non-desensitized cells. It should be noted that the band intensity of the antigen-driven syk phosphorylation was 0.30 of the anti-IgE response for the two experiments where they were compared and yet histamine release of 45% for antigen and 34% for anti-IgE. It should also be noted that when BPO-HSA was used to desensitize the cells, the resulting cells responded to further stimulation with anti-IgE antibody (85% of the non-desensitized response), as expected if the desensitization was primarily specific in character. As noted above, there was less recovery of syk during immunoadsorption when using desensitized cells. This difference in recovery only occurred with the cells were desensitized with anti-IgE antibody. Since the data on syk phosphorylation was normalized for differences in syk mass (as determined by stripping and re-probing the blots with anti-syk antibody), the data in figure 4 disguise the fact that the 4G10 blots show lower band intensities for the cells desensitized with anti-IgE antibody. Without normalization, the 4G10 band intensities for desensitized cells at the 5' rechallenge point were 61±9% of non-desensitized cells and for the 30' rechallenge point, 31±6% of the non-desensitized cells.

Inhibitors of early tyrosine kinases

The foregoing studies indicated that whatever process caused desensitization, it altered events that both preceded and followed syk phosphorylation. We were interested in whether desensitization required the same early signaling events as secretion, i.e., the activation of lyn and syk kinases. A pharmacological test of the involvement of syk kinase is not feasible at this time because we have found that picatannol, a putative syk inhibitor, does not appear to act on syk kinase in human basophils (manuscript in preparation). However, we have recently found that the src family kinase inhibitors, PP1 and PP2, were effective inhibitors of IgE-mediated histamine release and the phosphorylation of both lyn and syk (Lavens-Phillips *et al* manuscript submitted for publication).

We reasoned that inhibition of lyn kinase should provide a test of whether this type of early signaling is also necessary for desensitization. We have therefore examined the ability of PP1 to inhibit IgE-mediated desensitization.

Experimentally, in order to determine the effect of these inhibitors on the desensitization phase alone the compound must be effectively washed out of the cell with either a large dilution or a washing step (using centrifugation). Therefore, the experimental design is to stimulate the cells in the absence of extracellular calcium (desensitization phase)± inhibitor, wash the cells and rechallenge in the presence of extracellular calcium but in the absence of inhibitor. The requirements for being able to wash out the drug used during the desensitization phase of the experiment were relatively strict, since PP1 and PP2 are good inhibitors of secretion and would therefore interfere with the ability to examine the extent of desensitization by measuring subsequent histamine release. Thus, it was important to demonstrate that the washing step was effective even for weak stimulation during the re-challenge phase. We centrifuged the cells twice following the desensitization phase. The cells were divided as described in the methods section and one set of cells were treated with drug for the relevant period of time, washed and re-challenged with serial dilutions of the stimulus (anti-IgE antibody or antigen). Ideally, challenge of these so treated cells with any concentration of stimulus would be no different than cells not treated with drug. This was generally found to be the case. Figure 5a demonstrates that PP1 could partially reverse desensitization induced by anti-IgE antibody. We also examined three other IgE-mediated stimuli. Basophils which had been sensitized with either BPO-specific IgE were stimulated with either BPO-HSA or BPO2, whilst cells which were sensitized with gp120-specific IgE were stimulated with gp120-OVA. When these stimuli were used, PP1 also caused a reversal of desensitization; in some instances desensitization that resulted in a response that was 10% of the non-desensitized response was reversed to the point of being 90% of the non-desensitized response. On average, however, reversal of desensitization remained partial with no clear pattern of differences among the various stimuli. In one experiment, PP2 was found to cause a similar reversal of desensitization.

Role of PKC in desensitization

The PP1 studies indicated that early signaling through the canonical lyn signaling pathway was required for desensitization. Consequently, events normally considered downstream of these early steps could be involved in desensitization. One such element is the activation of PKC. PKC has been speculated to down-regulate IgE-mediated release and we have previously reported that the newer selective inhibitors of PKC, Bis II and Ro-31-8220 initiate a modest enhancement of IgE-mediated release, suggesting that these compounds may inhibit PKC activity that is involved in the down-regulation of the IgE-mediated response (9). These two drugs appear to relatively selective inhibitors of PKC and which can be effectively washed from the cells after the desensitization phase of the experiments. We have therefore used these PKC inhibitors to determine if PKC plays a role in the desensitization process in human basophils. In several pilot experiments, Bis II and Ro-31-8220 were first shown to be effectively removed from the cells by a washing step. In these experiments, the cells were first incubated with either 400 nM Bis II or 500 nM Ro-31-8220 for 15 minutes. At this time point the cells were then washed once prior to challenging with PMA without the further addition of either Bis II or Ro-31-8820. Our results indicate that there was little of no inhibition of the PMA-induced release (compared with cells not treated with Bis II or Ro-31-8820 and in contrast to cells were Bis II or Ro-31-8220 was added during stimulation with PMA), data not shown. Therefore, from these results Bis II and Ro-31-8220 could be included in the desensitization phase without significantly affecting the final challenge phase.

We next extended these initial studies to investigate the effect of these PKC inhibitors on desensitization induced by either polyclonal anti-IgE antibody or antigen. Human basophils were resuspended in PAG with 5 μM EDTA in the presence or absence of 500 nM Ro-31-8220 for 10 minutes. Anti-IgE (0.1 μg/ml) antibody was then added and the reaction allowed to proceed for 20 to 40 minutes (total volume at this stage was 50 μl). The reaction was then continued by the

addition of 0.95 ml of buffer containing calcium and magnesium at 1 mM (PAGCM) and where appropriate, enough anti-IgE antibody to result in a final concentration of 0.1 μg/ml. The reaction was then allowed to proceed for a further 45 minutes after which time histamine release was determined. Figure 1a shows the results for cells desensitized with anti-IgE antibody for the two time periods, 20 and 40 minutes. The non-desensitized response (cells incubated for 20 or 40 minutes in the absence of drug or anti-IgE during the calcium free phase) to anti-IgE in the absence of drug was 58% (column 1). Desensitization (treatment of the cells with anti-IgE antibody in the calcium-free phase) for 20 and 40 minutes in the absence of drug decreased histamine release to 23% and 15% respectively (columns 3 and 7). This decrease in histamine release during the desensitization phase was unchanged when Ro-31-8820 was also included (columns 4 and 8). Since these inhibitors have marginal effects on IgE-mediated release, their effectiveness was assessed by challenging cells with 30 ng/ml PMA. The results are shown in figure 1a and indicate that Ro-31-8220 effectively inhibited PKC induced histamine release from human basophils (compare column 13 to columns 9 and 11). In addition, the drug effect was effectively washed or diluted away as the PMA response under these conditions was 60-75% of the response in cells not treated with drug (compare columns 10 and 12 to columns 9 and 11 respectively). Together these data in figure 1a indicate that Ro-31-8220 has no effect on the rate or extent of desensitization measured this way. A similar series of experiments using Bis II resulted in the same conclusion (data not shown).

Desensitization has two components, the desensitization that may be specific for the antigen used to desensitize the cells or it may alter the response of the cells to other non-crossreacting antigens (10, 11). To examine both specific and nonspecific desensitization, two additional criteria need to be satisfied. The cells need to be sensitized with two non-crossreacting antigen-specific IgE antibodies and preferably, it is useful to dissociate the antigen used to desensitize the cell from the cell surface prior to challenge with the second non-crossreacting antigen. The use of anti-IgE antibody as a stimulus precludes an analysis of these two processes since it potentially crosslinks

all cell surface IgE antibody. Therefore, basophil preparations were sensitized with a mixture of penicillin-specific and gp120-specific IgE (see methods section) prior to the experiment. The sensitized cells, suspended in PAG with 5 μM EDTA were split in to four pots, two of which were not desensitized±Ro-31-8220 (500 nM) and two of which were desensitized with BPO-HSA (500 ng/ml)±RO-31-8220 (500 nM). All four pots were incubated for 60 minutes. The pots were then centrifuged and the cell pellets resuspended in PAG and distributed to tubes for challenge. The cells which were to be challenged with gp120-OVA, PMA or PAGCM (spontaneous release controls) were first treated for 5 minutes with 0.1 mM BPO-EACA to dissociate any bound BPO-HSA and then the stimuli were added in buffer containing enough calcium and magnesium to result in 1 mM final concentrations. For cell rechallenged with BPO-HSA, the monovalent hapten wasn't included. This protocol differs from that for the experiments shown in figure ? in that the effective dilution of the drug should have been greater since the cells were centrifuged to remove the initial desensitization buffer. Furthermore, the BPO-EACA was required to eliminate the influence of any residual signaling by the BPO-HSA used in the desensitization of the experiment (although it was found to be minimal). It can be seen in Figure 1B that the response to BPO-HSA was completely ablated following 60 minutes of desensitization (compare column 3 with column 1). Ro-31-8220 had no effect on this desensitization (compare column 4 with column 3). Thus Ro-31-8220 had no effect on specific desensitization. likewise, there was no effect of Ro-31-8220 on non-specific desensitization. Comparing column 7 with column 5, it can be seen that there was partial non-specific desensitization, i.e. pretreatment with BPO-HSA caused partial inhibition of the cell's response to gp120-OVA (≈25%). Ro-31-8220 did not change the extent of non-specific desensitization (compare columns 7 and 8). The PMA controls for this experiment are shown in the right half of panel B. As observed in the experiments shown in figure 1A, Ro-31-8220 effectively inhibited the PMA response (column 13) and there was partial recovery (≈76%) from this inhibition after removal of the drug by washing the cells (compare columns 10 and 9 or 12 and 11).

Discussion

These studies focused on syk phosphorylation as an indicator of the activity of this apparently essential signaling molecule. Whether it is a proper assumption to use tyrosine phosphorylation of syk kinase as an indicator of its activity, the results differentiated between the characteristics of its phosphorylation state when comparing cells undergoing specific vs. nonspecific desensitization. Antigen-driven release, in sensitized cells, resulted in transient phosphorylation of syk kinase. In contrast, stimulation with anti-IgE antibody led to a sustained phosphorylation state for syk. Stimulation with anti-IgE antibody should result in aggregation of all cell surface IgE. A variety of prior studies suggest that desensitization induced by this stimulus should be dominated by events leading to nonspecific desensitization. However, the characteristics of desensitization with anti-IgE antibody can not be assessed by rechallenge with specific antigens because anti-IgE antibody, by its nature, will aggregate all surface IgE. Using cells sensitized with mouse IgE, we demonstrated that indeed, anti-IgE antibody induced complete nonspecific desensitization. These studies also showed that antigen stimulation resulted in specific desensitization, as assessed by subsequent histamine release. Therefore, one interpretation of the differences between the characteristics of syk phosphorylation following stimulation with antigen vs. anti-IgE antibody is that we are observing the differences between specific and nonspecific desensitization. Previous studies have indicated that nonspecific desensitization is actually a combination of events leading to specific desensitization as well as events leading to nonspecific desensitization. The working model is that specific desensitization always occurs while the strength of signal determines the relative activation of the nonspecific process. Furthermore, the working hypothesis states that if the signal is strong enough, the dominant effect on secretion comes from the process of nonspecific desensitization. These data support this hypothesis. This can be seen in the 30 minute measurements of syk phosphorylation following 60 minutes of desensitization with anti-IgE antibody. Although there is no blunting of early syk phosphorylation, the overall kinetic curve is blunted. At the 30 minute point, the cells had actually been experiencing aggregation that persisted for 90 minutes. Some

reduction of syk phosphorylation would be expected by this time. In addition, previous studies have shown that the calcium response is ablated following 60 minutes of desensitization. The fact that syk phosphorylation is somewhat sensitive to the presence of extracellular calcium raises the possibility that a calcium response indirectly alters the ability of the early stages of the reaction to lead to syk phosphorylation. Therefore, in desensitized cells where the cytosolic calcium response is blunted, one might expect to find less syk phosphorylation.

For most donors, the kinetics of histamine release following partial desensitization is faster than untreated cells: by 10 minutes, secretion is largely complete. Previous interpretations of this observation were based on the fact that binding of anti-IgE antibody takes some time so that desensitized cells receive calcium having already established a steady state level of cell surface aggregation. We also know that restoring extracellular calcium to cells incubated for some time in its absence results in a very transient overshoot of basal calcium levels. Together, these two circumstances may accelerate the rate of histamine release. The time frame of histamine release lies in the first 10-15 minutes of the reaction in partially desensitized cells so that its relative absence even while syk phosphorylation is not blunted indicates that some form of desensitization operates between syk phosphorylation and the calcium response. We do not yet know if the modification lies with syk itself. It is now recognized that there are 7-8 syk tyrosine phosphorylation sites, at least one of which leads to inhibition of syk kinase activity. It is therefore conceivable that 4G10 detection of phosphorylation may not indicate the activity of syk kinase. However, in studies prepared for another manuscript, we will be showing that Shc phosphorylation is sustained in cells stimulated with anti-IgE antibody. Shc is thought to be an immediate substrate for syk, so these data indicate that in vivo, syk activity is maintained and therefore that its phosphorylation state reflects its activation state under these conditions. The data also indicates that syk is more difficult to immunoadsorb when obtained from desensitized cells. It is possible that this also indicates a different phosphorylation state for syk which interferes with its binding to solid phase 4D10 anti-syk antibody or that there is a protein bound to syk which interferes with its adsorption to the solid

phase 4D10. We are currently trying to determine the cause of this phenomenon. But whatever its cause, the results could be re-interpreted without normalization for recovery and even with this analysis, there remains a marked difference in what happens during stimulation with anti-IgE antibody vs. antigen.

The transient phosphorylation of syk during antigen stimulation and the ablation of syk phosphorylation in antigen-desensitized cells indicates that specific desensitization does modify an event earlier than syk phosphorylation. The events immediately preceding syk phosphorylation include only the changes in lyn activity and phosphorylation of the receptor, presumably by lyn. Recruitment of phosphatases to the receptor could cause these effects or sequestration of the receptor or lyn from the normal membrane signaling environment could have this effect. These possibilities are currently being studied.

The pharmacological studies indicate that both specific and nonspecific desensitization require the activity of lyn kinase. This result does not readily preclude the two possibilities for specific desensitization discussed above, but it does indicate that both activation and desensitization have similar early signaling requirements. The pharmacological experiments also indicate that one possible downstream regulator of activation, the activation of PKC, does not play a role in either specific or nonspecific desensitization. Although we have not found direct evidence for PKC activation during IgE-mediated release, previous studies of the effects of either Bis II or Ro 31-8220 noted that some concentrations of the two drugs caused modest enhancement of secretion, especially IL-4 secretion. Thus, it is surprising that the two drugs had no effect on desensitization.

In summary, these data have found a point in the signal transduction cascade, syk phosphorylation, that differentiates between activation that is typically associated with specific desensitization and activation that often leads to nonspecific desensitization. In forthcoming studies we hope to narrow down the point at which nonspecific desensitization appears to act. We are also

investigating the phosphorylation of FceRI subunits β and γ in order to determine what happens to these two components during specific desensitization.

Footnotes:

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[2]We have relied on past experience to make conclusions regarding the occurence of specific and nonspecific desensitization in the cells used for these studies. Basophils generally possess ≈5000 unoccupied receptors and greater than 100,000 receptors occupied with IgE of unknown specificity. Previous studies have also indicated that crosslinking less than 5000 cell surface receptors does not lead to nonspecific desensitization. This has been experimentally demonstrated a number of times. Testing for nonspecific desensitization requires that two non-crossreacting antigen-specific IgE antibodies be present on the cell surface. Because polyclonal anti-IgE antibody has the potential to aggregate all cell surface IgE, this stimulus could simply lead to a form of specific desensitization that appears nonspecific because all cell surface IgE is aggregated. Nevertheless, based on the number of crosslinks formed by this stimulus, it should lead to desensitization that is dominated by the nonspecific process (see discussion). To verify that desensitization with anti-IgE antibody is indeed nonspecific in character, we sensitized basophils with DNP-specific mouse IgE and desensitized the cells with either DNP-HSA or the goat polyclonal anti-hIgE antibody normally used in our experiments. Under these conditions, complete desensitization with DNP-HSA (60 minutes of desensitization with an optimal concentration of DNP-HSA) had little effect on release induced by anti-IgE antibody while desensitization with anti-IgE antibody ablated the response to DNP-HSA as well. While there should be no cross-reactivity between the goat polyclonal anti-hIgE and the DNP-specific mouse IgE used for sensitization, we examined this assumption by sensitizing RBL-SX38 cells with the DNP-specific mouse IgE (near saturating conditions, 1 μg/ml for 1 hour at 37°C) and stimulated with either DNP-HSA or goat anti-hIgE antibody. No secretion was observed following anti-hIgE antibody while typical levels

of secretion (25-30%) followed challenge with DNP-HSA. Sensitizing the cells with 30 fold less DNP-specific IgE resulted in cells that continued to respond well to DNP-HSA indicating that the absence of a response to anti-hIgE antibody indicates that if it is able to crosslink mouse IgE, it does so with very poor efficiency. Likewise, sensitizing these cells with PS myeloma led to histamine release when the cells were stimulated with goat polyclonal anti-IgE antibody but not DNP-HSA. These results provide supportive evidence that desensitization with anti-IgE antibody results in a process which has a strong component of nonspecific desensitization.

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Figure Legends

Figure 1: Kinetics of syk phosphorylation in the presence and absence of extracellular calcium in basophils stimulated with an optimal concentration of anti-IgE antibody. Panel A shows a composite of three experiments where basophils were challenged with 0.2 μg/ml of anti-IgE antibody and cells were harvested at the various time shown and analyzed for syk phosphorylation of syk after immunoadsorption with anti-syk/Staph G beads. For two experiments, time points 3-5', 15' and 120 minutes were examined and for one experiment, the one hour time point was included. The insert for panel A shows a representative Western blot of syk phosphorylation as determined by the 4G10 anti-phosphotyrosine probe. Histamine release averaged 35%. Panel B shows the ratio of syk phosphorylation determined at 5' post-stimulation for cells challenged in the presence or absence of extracellular calcium in those preparations where the two conditions were examined using the same preparation of cells. Panel C shows a composite of three experiments for cells challenged with 0.2 μg/ml of anti-IgE antibody in the absence of extracellular calcium (PAG buffer with 5 μM EDTA). Two experiments included the 15 minute time point and one experiment included only the 5 and 60 minute time points. The insert shows a representative Western blot for these experiments, the top blots using the 4G10 probe and the bottom blots anti-syk probe after stripping the top blots.

Figure 2: Kinetics of syk phosphorylation in the presence and absence of extracellular calcium in basophils stimulated with an optimal concentration of BPO-HSA. Panel A shows a composite of three experiments where basophils were challenged with 0.5 μg/ml of BPO-HSA and cells were harvested at the various time shown and analyzed for syk phosphorylation of syk after immunoadsorption with anti-syk/Staph G beads. The

cells had been sensitized with penicillin-specific IgE (see methods). For all three experiments, time points 5' and 60 minutes were examined and for one experiment, the two hour time point was included. The insert for panel A shows a representative Western blot of syk phosphorylation as determined by the 4G10 anti-phosphotyrosine probe in the top row of blots and the anti-syk probe in the bottom row of blots. Histamine release averaged 54%%. Panel B shows the ratio of syk phosphorylation determined at 5' post-stimulation for cells challenged in the presence or absence of extracellular calcium in those preparations where the two conditions were examined using the same preparation of cells. Panel C shows a composite of three experiments for cells challenged with 0.5 μg/ml of BPO-HSA in the absence of extracellular calcium (PAG buffer with 5 μM EDTA). All three experiments examined the 5' and 60' time points, two experiments included the 15 minute time point and one experiment added the 120 minute time point. The insert shows a representative Western blot for these experiments, the top blots using the 4G10 probe and the bottom blots anti-syk probe after stripping the top blots. The open circles in panels A and C show the extent of syk phosphorylation after the addition of BPO-EACA to cells at the times represented by the start of the dotted line segments. These data points were included in one experiment for the additions at 5 minutes and in two experiments for the additions at 60 minutes.

Figure 3: Representative Western blots for experiments where the cells were first desensitized with anti-IgE antibody or BPO-HSA. Panel A shows an experiment where cells were desensitized for 60 minutes followed by the addition of calcium for 5 additional minutes before harvesting; anti-IgE at 0.2 μg/ml and BPO-HSA at 0.5 μg/ml. Descriptive line (1) indicates the presence of the stimulus in the desensitization phase (the first 60 minutes in PAG buffer) and descriptive line 2 indicates the presence of the stimulus in the 5' calcium phase. The top row of blots shows the results for the 4G10

probe and the bottom row of blots shows the results for the anti-syk probe. After obtaining the 4G10 data, the blots were stripped and re-probed with the anti-syk probe. Panels B and C show experiments were 0.2 μg/ml of anti-IgE antibody was used to desensitize the cells for 60 minutes. Following the addition of extracellular calcium, cells were harvested at 5' or 30'. Line 1 of the descriptive text indicates the presence of stimulus during the desensitization phase and line 2 indicates the presence of stimulus during the calcium phase. The top row of blots for each panel shows the results of the 4G10 probe and the bottom row the anti-syk probe.

Figure 4: Summary of the results of desensitization experiments. Scanned blots for both the 4G10 and anti-syk probes were analyzed as the ratio of band intensities for desensitized/non-desensitized cells at the time shown. Band intensities for the 4G10 probe were normalized using the band intensities for the anti-syk probe to account for differences in the amount of syk loaded. The left side of the figure shows results where 0.2 μg/ml anti-IgE antibody was used to desensitize the cells for 60 minutes. There were different numbers of experiments done for the various times examined after the addition of extracellular calcium; n=6 for the 5' time point, n=1 for the 10 minute time point and n=4 for the 30 minute time point. Four of the six experiments examined both the 5 and 30 minute time points and one experiment examined 5, 10 and 30 minutes. Histamine release was examined in experiments. The middle two stripped bars show data from three Western blots where whole cell lysates were used without immunoadsorption. The p72 region was analyzed for desensitized and non-desensitized cells. The right two bars of the figure show results for cells sensitized with penicillin-specific IgE and desensitized for 60 minutes with 0.5 μg/ml BPO-HSA. Five minutes after the addition of extracellular calcium, the cells were harvested for analysis of syk phosphorylation.

Figure 5: Reversal of desensitization in cells co-incubated with PP1. Panels A and B separate the results into preparations the were desensitized with (0.2 μg/ml) anti-IgE antibody or preparations that were first sensitized with antigen-specific IgE and desensitized with the relevant antigen. The results in panel B represent a mix of experiments where cells were sensitized with either BPO-specific IgE or gp120-specific IgE. For those experiments where the cells were sensitized with BPO-specific IgE, some included desensitization with BPO2 and others used BPO-HSA. BPO2 concentrations were 0.5 nM, BPO-HSA concentrations were 0.5 μg/ml and gp120-OVA concentrations were 50 ng/ml. The open circles in panel B represent the results for experiments where desensitization was done with BPO2. Otherwise, the results are not distinguished by different symbols. Histamine release from non-desensitized cells averaged 45% for anti-IgE antibody, 50% for BPO-HSA, 28% for BPO2 and 43% for gp120-OVA. See methods for specific details of the incubations.

Figure 6: No reversal of desensitization in cells treated with PKC inhibitors, BisII or Ro-31-8220. Panel A (n=3) shows results for cells desensitized with anti-IgE antibody (0.2 μg/ml) for times that do not result in complete desensitization. The right side of the panel shows the control data (see text) designed to assess the effectiveness of the drug and the success of the drug wash out step (see text). Panel B (n=3) shows the results for cells sensitized with both BPO-specific and gp120-specific IgEs, desensitized with 0.5 μg/ml BPO-HSA in the presence or absence of Ro-31-8220 and rechallenged with either BPO-HSA (0.5 μg/ml) or gp120-OVA (50 ng/ml). The right side of the panel provides the controls to determine the effectiveness of the drug and the success of its washout.

Figure 1

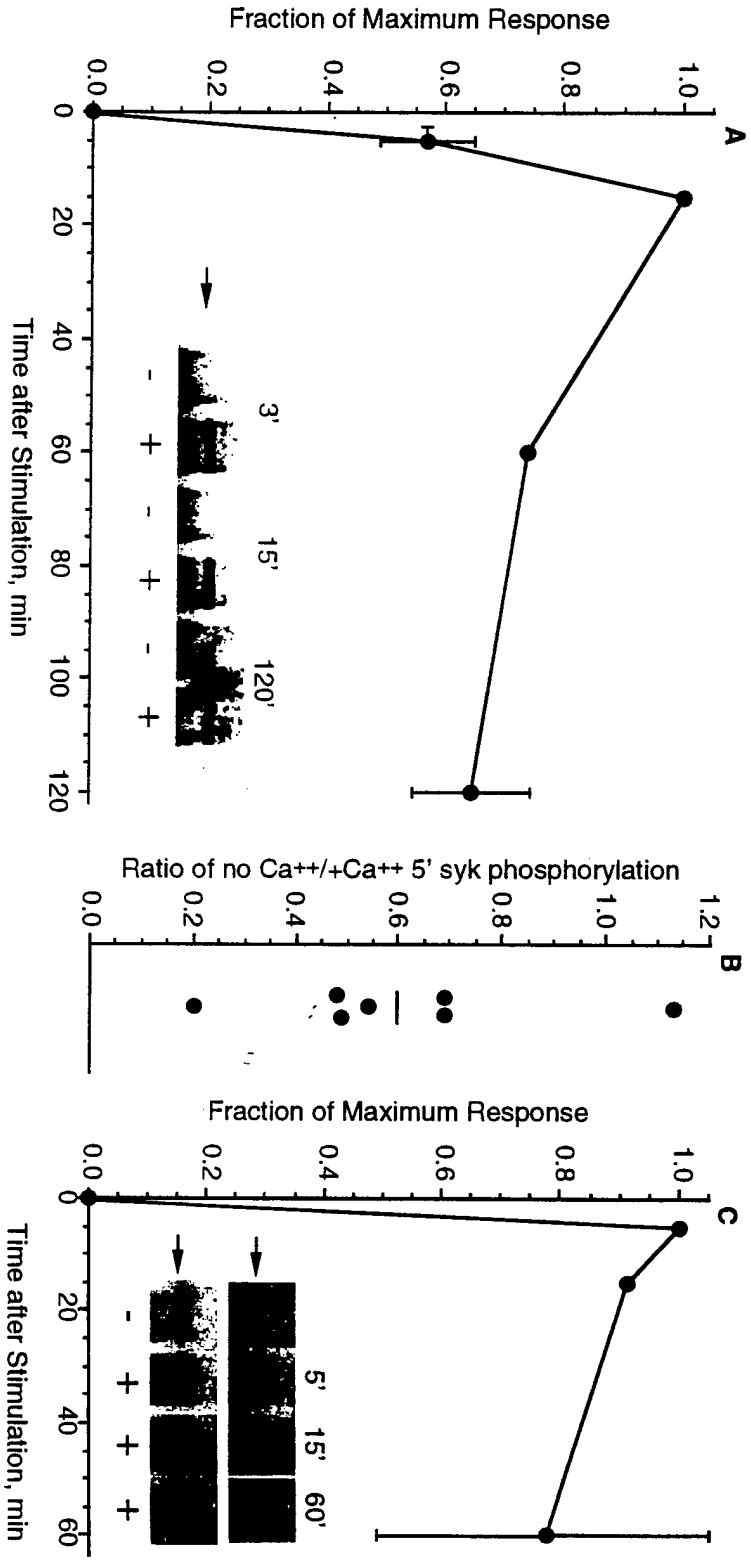

Fraction of Maximum Response
0.0
0.2
0.4
0.6
0.8
1.0
A
0
20
40
60
80
100
120
Time after Stimulation, min
3'
15'
120'
-
+
-
+
-
+
Ratio of no Ca++/+Ca++ 5' syk phosphorylation
0.0
0.2
0.4
0.6
0.8
1.0
1.2
B
Fraction of Maximum Response
0.0
0.2
0.4
0.6
0.8
1.0
C
0
20
40
60
Time after Stimulation, min
5'
15'
60'
-
+
+
+

Figure 2

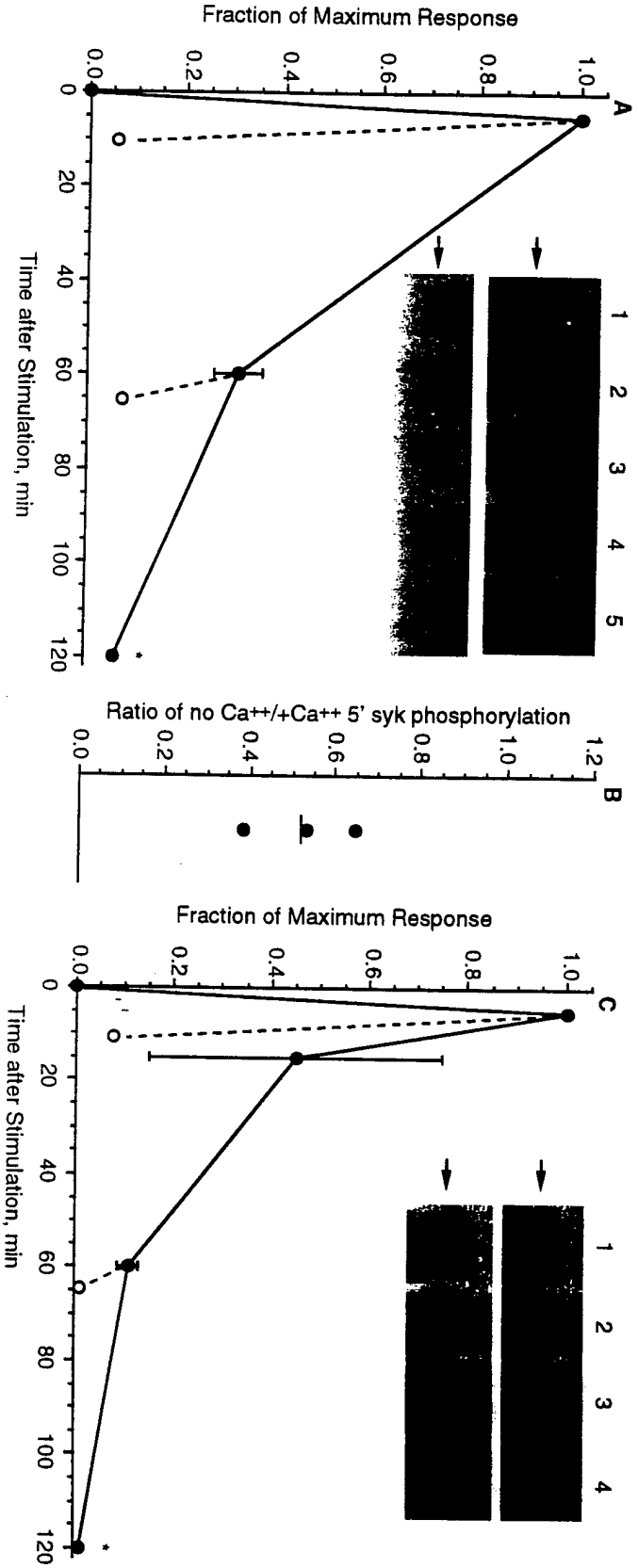

Fraction of Maximum Response
0.0
0.2
0.4
0.6
0.8
1.0
A
Time after Stimulation, min
0
20
40
60
80
100
120
1
2
3
4
5
Ratio of no Ca++/+Ca++ 5' syk phosphorylation
0.0
0.2
0.4
0.6
0.8
1.0
1.2
B
Fraction of Maximum Response
C
Time after Stimulation, min
1
2
3
4

Figure 3

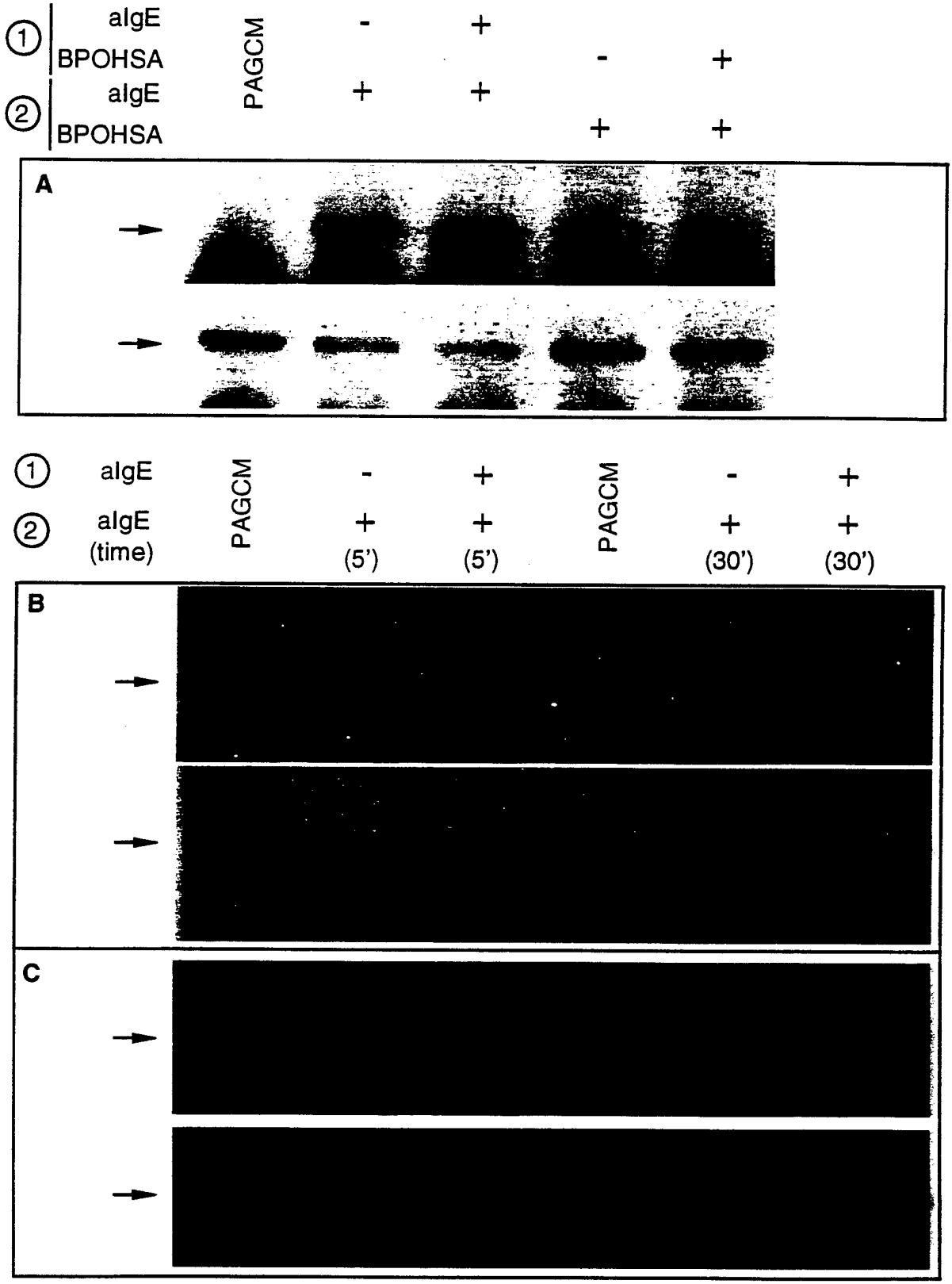

① aIgE
BPOHSA
② aIgE
BPOHSA
PAGCM
-
+
+
+
-
+
+
+
A
① aIgE
② aIgE (time)
PAGCM
-
+
+ (5')
+ (5')
PAGCM
-
+
+ (30')
+ (30')
B
C

Figure 4

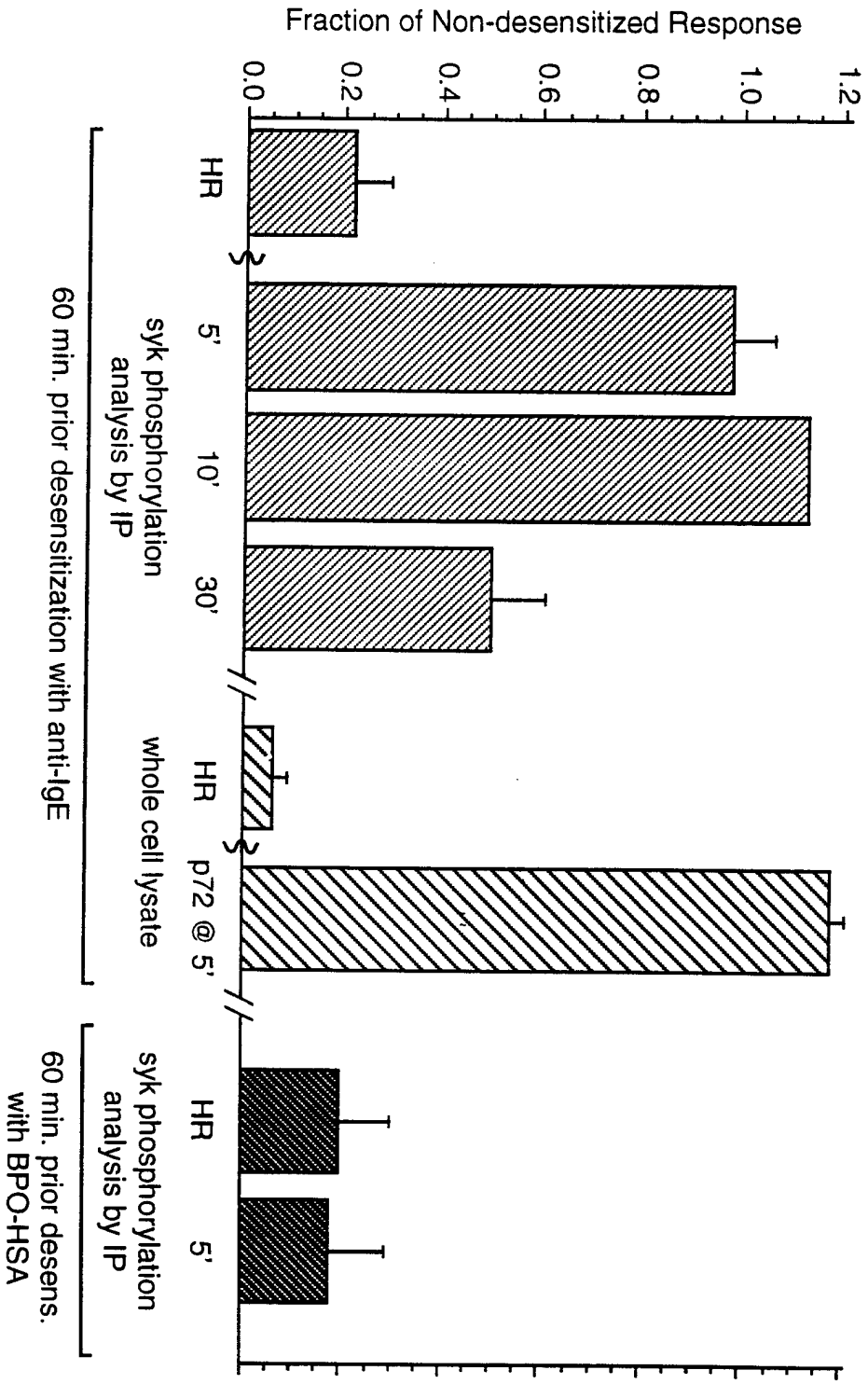

Fraction of Non-desensitized Response
0.0
0.2
0.4
0.6
0.8
1.0
1.2
HR
5'
10'
30'
HR
p72 @ 5'
HR
5'
syk phosphorylation analysis by IP
whole cell lysate
syk phosphorylation analysis by IP
60 min. prior desensitization with anti-IgE
60 min. prior desens. with BPO-HSA

Figure 5

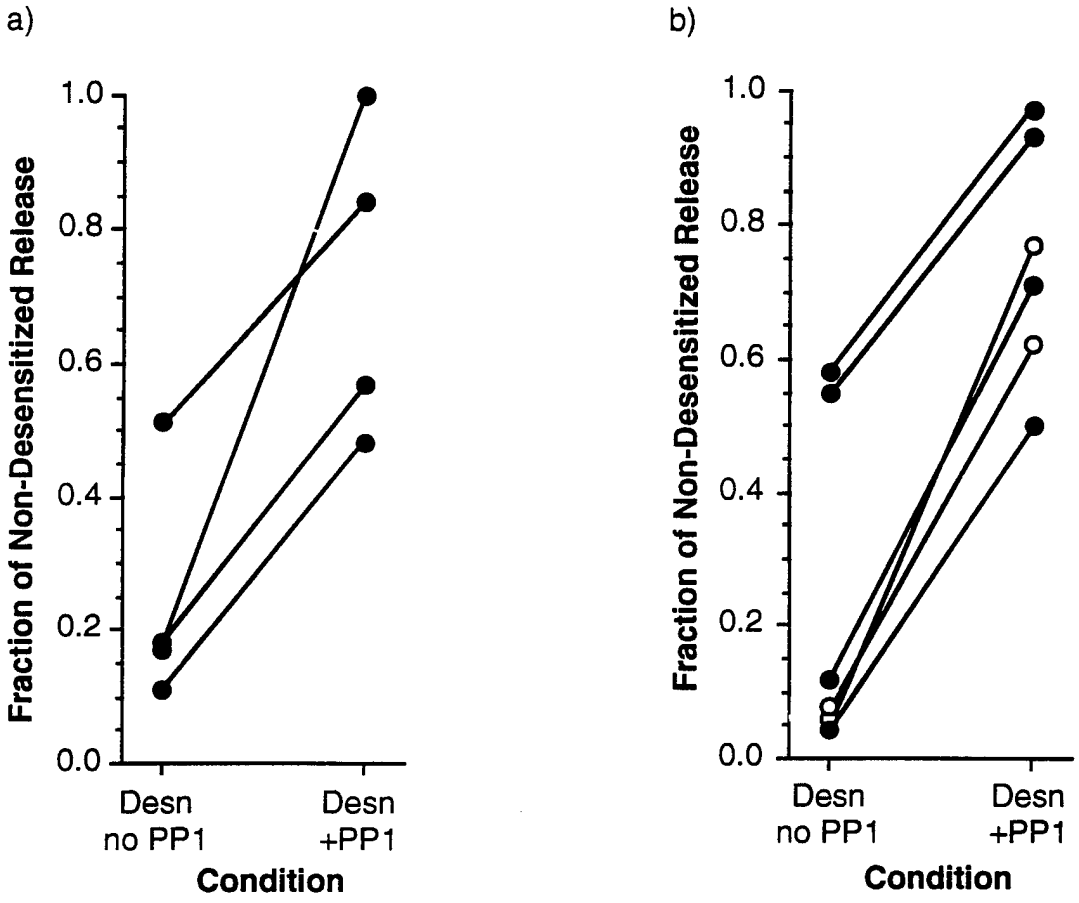
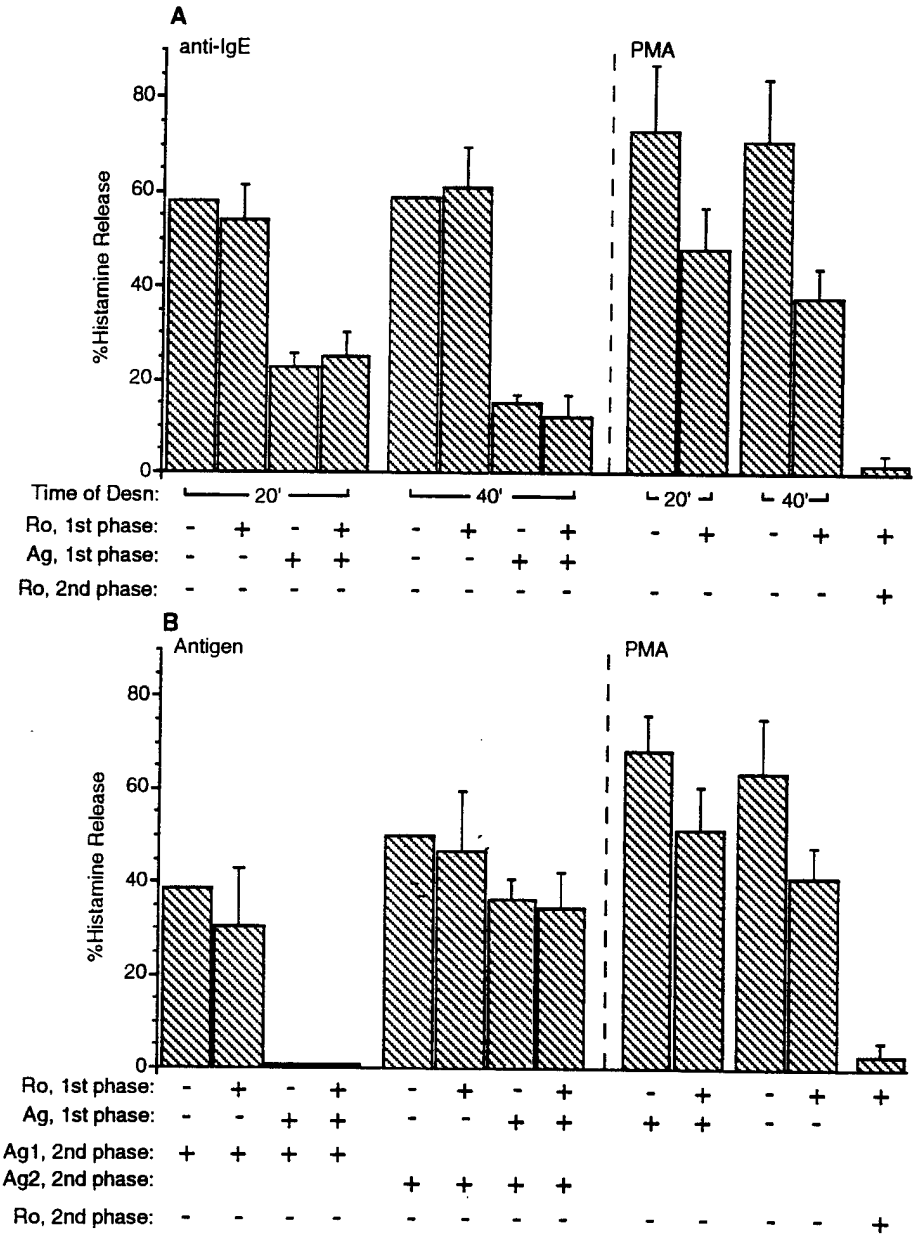

a)
b)
Fraction of Non-Desensitized Release
1.0
0.8
0.6
0.4
0.2
0.0
Desn
no PP1
Desn
+PP1
Condition

Figure 6


A
anti-IgE
PMA
%Histamine Release
80
60
40
20
0
Time of Desn:
20'
40'
20'
40'
Ro, 1st phase: - + - + - + - + - + - + +
Ag, 1st phase: - - + + - - + +
Ro, 2nd phase: - - - - - - - - - - - - +
B
Antigen
PMA
%Histamine Release
80
60
40
20
0
Ro, 1st phase: - + - + - + - + - + - + +
Ag, 1st phase: - - + + - - + + + + - -
Ag1, 2nd phase: + + + +
Ag2, 2nd phase: + + + +
Ro, 2nd phase: - - - - - - - - - - - - +

The effect of PP1 and PP2 on IgE-mediated signaling events in human basophils.

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Running title: The effect of PP1 and PP2 on signaling events in human basophils

Abbreviations: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; fMLP, formyl methionyl-leucyl phenyalanine; FcεRI, high affinity IgE receptor; HSA, human serum albumin; HR, histamine release; IgE, immunoglobulin E; IL-4, interleukin 4; ITAM, immunoreceptor tyrosine activation motif; kDa, kilodalton; LTC_4. leukotriene C4; PMSF, phenylmethylsulphonyl fluoride PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol myristate acetate; PP1/2, pyrazolopyrimidine 1/2; RBL2H3, rat basophilic leukemia cell line; SDS, sodium dodecylsulphate; SH, src homology; SI, stimulation index; TBST, tris-buffered saline tween.

Introduction

Aggregation of the high affinity IgE receptor (FcεRI) on the surface of mast cells and basophils generates a number of biochemical events leading to degranulation and the release of various mediators including histamine [1, 2], arachidonic acid metabolites [3-5] and cytokines [6, 7]. The high affinity IgE receptor belongs to a family of multichain immune system receptors that includes the T-cell receptor (TCR), the mIg receptor of B-cells and the high (FcγRI) and low affinity (FcγRIII/CD16) Fc receptors for IgG. These receptors all lack intrinsic tyrosine kinase activity [8]. Instead, they recruit and activate cytoplasmic tyrosine kinases, which in turn phosphorylate tyrosine residues in characteristic receptor subunit sequences called immunoreceptor tyrosine activation motifs (ITAMs) [9-11].

The most detailed investigations into the molecular mechanisms involved in this process have been carried out in rodent mast cell lines, and many of the earliest steps have been characterized in these cells. Among the tyrosine kinases, which have been shown to be recruited upon cell activation, are members of the src and syk families. In particular, lyn, a member of the src family of tyrosine kinases has been shown in the rat basophilic leukemia cell line (RBL-2H3) to be preferentially associated with the unactivated β subunit of the IgE receptor [12], therefore making lyn an ideal candidate for directly causing receptor phosphorylation. Syk is a 72 kDa protein tyrosine kinase [13] and defines another family of cytoplasmic enzymes distinguished by the presence of two tandem SH2 domains [13]. Upon IgE crosslinking, syk has been shown to bind to the phosphorylated ITAMs present within the γ subunit of the FcεRI [14-17]. Recent studies have indicated that these two families of tyrosine kinases are also involved in IgE-mediated signaling in human basophils [18-20].

Recently, two src selective tyrosine kinase inhibitors, PP1 and PP2 have been described [21]. These tyrosine kinase inhibitors, unlike previously described tyrosine kinase inhibitors, inhibit the src tyrosine kinases, Lck and Fyn *in vitro* at concentrations significantly lower than those required

to inhibit Zap-70, JAK 2, the EGF-R kinase and protein kinase A [21]. In addition, PP1 has also been shown in the RBL-2H3 cell line to selectively inhibit lyn kinase activity without any effect on syk [22]. We have therefore utilized these src specific tyrosine kinase inhibitors to study their effect on IgE-mediated activation and degranulation of human basophils.

Materials and Methods

Materials

Formyl methionyl-leucyl-phenylalanine (fMLP), phorbol myristate acetate (PMA), calcium ionophore, ionomycin, piperazine N, N' bis 2 ethane sulphonic acid (PIPES), glucose, fetal calf serum, human serum albumin (HSA), bovine serum albumin (BSA), perchloric acid, sodium orthovanadate, benzamidine, aprotinin, phenylmethylsulphonyl fluoride (PMSF), sodium fluoride, 2-mercaptoethanol and nonidet P-40 were all purchased from Sigma Chemical Co. (MO, USA). RPMI 1640 supplemented with 25mM hepes and L-glutamine was bought from Biowhittaker (MO, USA) while gentamycin was obtained from Gibco BRL (NY, USA). Sodium dodecylsulphate (SDS), tween 20 and tris were purchased from Bio-Rad (NY, USA). Protein G sepharose and Percoll were purchased from Pharmacia Biotec (NJ, USA). 4-20% and 10% tris-glycine gels and 2x SDS sample buffer were bought from Novex (CA, USA) while biotinylated molecular weight markers were purchased from New England Biolabs (MA, USA). The rabbit anti-human p53/56lyn was purchased from Santa Cruz Biotechnology Inc. (CA, USA), while the mouse anti-human p53/56lyn was purchased from Transduction Labs (KY, USA). The mouse anti-phosphotyrosine monoclonal antibody, 4G10 was purchased from Upstate Biotechnology (NY, USA). Sheep anti-mouse Ig horseradish peroxidase, donkey anti-rabbit Ig horseradish peroxidase, streptavidin horseradish peroxidase conjugate, ECL western blotting detection agents and ECL hyperfilm were all purchased from Amersham (IL, USA). The tyrosine kinase inhibitors, PP1 and PP2 were purchased from Biomol (PA, USA). Goat anti-human IgE was prepared as described previously; the antibody used for these studies represented the IgG fraction

of goat serum prepared by DE-52 chromatography [23]. All other reagents used were of the highest grade available.

Buffers

PIPES buffer contained 25 mM PIPES, 110 mM NaCl and 5 mM KCl adjusted to pH 7.4 with 1 N HCl; PIPES-albumin-glucose (PAG) also contained 0.003% (w/v) human serum albumin (HSA) and 0.1% (w/v) glucose; PAGCM was supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$; elutriation buffer for the purification of human basophils contained 10% PIPES, 0.1% (w/v) glucose and 0.25% (w/v) bovine serum albumin (BSA). Lysis buffer contained 20 mM Tris (pH 7.8), 150 mM sodium chloride, 1% nonidet P-40, 5% glycerol, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM benzamidine, 1 μg/ml aprotinin. In the electrophoresis studies, 2x SDS sample buffer contained 0.5 M Tris-HCl, pH 6.8, 10% (w/v) SDS, 0.1% bromophenol blue, 20% glycerol, and 5% mercaptoethanol; TBST buffer contained 12 mM Tris base (pH7.5), 150 mM NaCl and 0.05% tween-20; running buffer contained 25 mM Tris base, 192 mM glycine and 0.1% SDS; transfer buffer contained 12 mM Tris base, 96 mM glycine and 20% methanol; stripping buffer contained 7M Guanidine.

Preparation of the tyrosine kinase inhibitors

PP1 and PP2 were both prepared as 10 mM solutions in DMSO and stored at -20° C. Prior to use the inhibitors were diluted to the required concentration in PAGCM. Control experiments confirmed that DMSO at these concentrations did not affect the release of histamine. In addition, the drugs were not cytotoxic as assessed by trypan blue exclusion.

Isolation of human basophils

Basophils were prepared from buffy coat cells, obtained from normal donors undergoing hemaphoresis, using counter current elutriation and Percoll density gradients [24]. For the immunoprecipitation studies the basophils were further purified using negative selection as described previously [25]. Final basophil purities were 5-20% for the mediator release studies and >90% for the immunoprecipitation studies. In all instances the contaminating cells consisted mainly of lymphocytes and monocytes.

Cell counting

Mast cells and basophils were stained with Alcian blue [26] and counted in a Spiers Levy hemocytometer.

Cell stimulation and preparation of cell lysate

Purified human basophils were resuspended in PAGCM prior to their stimulation with either 200 ng/ml anti-IgE, 10 ng/ml PMA, 1 μg/ml ionomycin or 1 μM fMLP. 100 μl aliquots were then removed at various time points and added to 900 μl of ice-cold PAGCM. The cell free supernatants were then removed for histamine analysis and the cell pellet lysed by the addition of 40 μl of 1x SDS sample buffer/10^6 cells. In some experiments, human basophils were incubated with either PP1 or PP2 for 10 minutes prior to stimulation. The phosphotyrosine containing proteins were then analyzed as described below.

Immunoprecipitation

Lysates from $2x10^6$ basophils per condition were incubated with protein G sepharose beads for 30 minutes at 4° C to remove any non-specific binding to the beads. After this time, the lysates were then incubated with 1 μg/ml rabbit anti-p53/56lyn prebound to protein G sepharose beads. After gentle rotation for 1 hour at 4° C, the beads were washed four times in ice-cold lysis buffer. The immunoprecipitated proteins were then eluted from the beads by boiling in 1x SDS sample buffer. Control experiments revealed that an irrelevent IgG antibody or mouse anti-lyn in the absence of lysate did not pull down lyn in the immunoprecipitates.

Blotting of proteins

Proteins were separated in a 4-20% tris-glycine gel under reducing conditions and electrotransferred on to a nitrocellulose membrane. The free binding sites were blocked by incubating the membrane overnight at 4° C with 4% BSA in TBST. The nitrocellulose membranes were then incubated with 0.5 μg/ml of the anti-phosphotyrosine monoclonal antibody, 4G10, in 1% BSA/TBST for 1 hour at room temperature. The membrane was then thoroughly washed with TBST prior to the addition of an anti-mouse horseradish peroxidase conjugate (1:0.5-$1x10^4$ dilution) for 1 hour at room temperature. After further extensive washing of the membranes with

TBST the phosphoproteins were visualized using Enhanced Chemiluminescence. The nitrocellulose membrane was exposed to ECL hyperfilm for 15 seconds to 2 minutes. In some experiments, the nitrocellulose membrane was stripped for 30 minutes at room temperature, then reprobed with 0.2 μg/ml mouse anti-human p53/56lyn. After exposure to chemiluminescence detection agents, the intensity of each band was determined using densitometric analysis. The increase in tyrosine phosphorylation of a relevant band was then determined as a stimulation index ie the increase in band density after anti-IgE induced stimulation compared with unstimulated cells. In all experiments, equal protein loading was confirmed by Coomassey Blue staining.

Histamine release

Histamine release was measured directly by removing a volume equivalent to $30x10^3$ basophils from the cell free supernatant. The total histamine content was determined by treating $30x10^3$ basophils with 200 μl of 8% perchloric acid. The volume of all the samples was then brought up to 1 ml with PAGCM. For the experiments investigating the effect of PP1 or PP2, impure preparations of human basophils (approximately $2x10^4$ per tube) were resuspended in PAGCM and incubated with drug for 10 minutes at 37° C. Anti-IgE (200 ng/ml) was then added and mediator release allowed to proceed for a further 45 minutes at 37° C. In some experiments basophils were challenged with either fMLP (1 μM) or two non-receptor mediated stimuli PMA (10 ng/ml) and ionomycin (1μg/ml). Histamine was determined by the automated method of Siraganian [27]

LTC4 release

100 μl aliquots of cell free supernatants (approximately $10x10^3$ basophils) were measured for LTC4 using a radioimmunoassay as described previously [5, 28].

IL-4 release

Experiments were done in 96 well flat bottomed microtiter plates (Corning Inc., Corning NY) using IMDM containing 5% heat inactivated FBS, 1x non-essential amino acids and 10 μg/ml gentamycin. Human basophils were added in 100 μl of IMDM per well (at a basophil density of approximately $1\text{-}2x10^5$ per well), brought to 37° C in a CO_2 incubator (5% CO_2), after which time

100 µl of IMDM with drug or DMSO was added. After a 15 minute preincubation with drug the cells were activated by adding anti-IgE (final concentration was 10-25 ng/ml). For harvesting, cultures were centrifuged after 4-16 hours and the cell free supernatants collected. IL-4 protein release was then determined using an in-house ELISA (3 pg/ml sensitivity) [29]. Basophil purities in these preparations were typically >80%.

Calculation of results

The histamine results were based on the mean of duplicate determinations and were expressed as percentage of total histamine a value that was obtained by lyzing the cells in 8% (wt/vol) perchloric acid. Results were corrected for spontaneous release from unstimulated cells (less than 5% during a 45 minute assay). Each experiment was repeated using cells from at least 4 different donors and the number of experiments (n) given in the figure legend. Results were compared using a non-parametric signed rank test; a value of $P<0.05$ was accepted as statistically significant.

Results

We first examined the effect of PP1 and PP2 on the tyrosine phosphorylation of p53/56lyn following stimulation of human basophils with anti-IgE. Purified human basophils were preincubated for 10 minutes in either the presence or absence of 10 µM PP1 or PP2 (a concentration that will be shown below to inhibit histamine release) then stimulated with anti-IgE for 5 minutes. The cell lysates were then subjected to immunoprecipitation with a rabbit anti-human p53/56lyn (refer to materials and methods section). Figure 1, shows a Western blot of immunoprecipitated p53/56lyn probed with the anti-phosphotyrosine monoclonal antibody 4G10. As this figure demonstrates, p53/56lyn was tyrosine phosphorylated following anti-IgE stimulation (compare lane 2 with lane 1). However, in the presence of 10 µM PP1 this increase in tyrosine phosphorylation of p53/56lyn was completely ablated (compare lane 3 with lanes 1 and 2). Similar effects were also observed in the presence of 10 µM PP2 (lane 4). We found equivalent inhibition of IgE-mediated p53/56lyn phosphorylation by PP1 and PP2 (compare lane 4 with lane 3). It should also be noted that both PP1 and PP2 inhibited the basal lyn phosphorylation observed in

unstimulated basophils (compare lanes 3 and 4 with lane 1). The Western blot was stripped of anti-phosphotyrosine and reprobed with mouse anti-human p53/56lyn to ensure equal protein loading in each lane, see figure 1b.

We extended these initial experiments to examine the causal effect of p53/56lyn inhibition by either PP1 or PP2 on downstream signaling events in human basophils. Purified human basophils were stimulated with anti-IgE in the presence or absence of PP1 (10, 3 and 1 μM) for 5 minutes. The reaction was then terminated and the whole cell lysate examined by Western blotting for tyrosine phosphorylated proteins using the anti-phosphotyrosine antibody, 4G10. As figure 2 indicates, anti-IgE induced the increase in tyrosine phosphorylation of several proteins in the 130, 120, 75-65, 56/53, 40 and 26 kDa range (compare lanes 1 and 2). The phosphorylation of all substrates were significantly reduced by PP1 with an IC_{50} of ~2 μM (as measured by quantitating the p72 band from 3 separate experiments). This inhibition of tyrosine phosphorylation was not due to unequal protein loading as figure 2b demonstrates with an anti-syk blot. PP2 caused similar inhibition of anti-IgE induced tyrosine phosphorylation (data not shown).

Stimulation of human basophils or mast cells through the high affinity IgE receptor leads to cell activation and the release of various mediators including histamine , LTC_4 and IL-4 [1, 3, 7]. Based on the putative role of lyn kinase in the first step of the signaling cascade, PP1 and PP2 should inhibit the secretion of all mediators. As figure 3 indicates, PP1 was a potent inhibitor of anti-IgE induced histamine release from human basophils with an IC_{50} of 3 μM. At 10 μM the inhibition by PP1 was 94±3% (n=5, P=0.04) which was not exceeded at 100 μM (figure 3). In contrast, PP1 had no effect on histamine release induced by another receptor mediated stimuli, fMLP, see figure 3. At 10 μM PP1, the inhibition was 2±1% (n=5). In order to gain some insight in to the specificity of PP1 on anti-IgE mediated histamine release we have also assessed it's effects on two non-receptor mediated stimuli, PMA and ionomycin. Our results indicate that there

was a significant inhibition of histamine release at the highest concentrations of PP1 tested (30 and 100 μM), though at 60 μM the IC_{50} was 20 fold greater (data not shown).

As figure 4 indicates, PP2 also caused a significant inhibition of anti-IgE induced histamine release from human basophils with an IC_{50} value of 3 μM. At 10 μM PP2 the inhibition was 95±1% (n=9, P=0.01) and was not exceeded at 30 μM (figure 4). Interestingly, lower concentrations of PP2 caused a significant enhancement of anti-IgE induced histamine release. At 0.3 and 0.1 μM PP2, anti-IgE induced histamine release was increased from 53±6% to 71±6% and 68±5% respectively (n=9, P=0.008 and 0.04 respectively), see figure 4. In contrast to anti-IgE induced histamine release, PP2 had no significant effect on fMLP induced histamine release except at 30 μM, see figure 4. We also investigated the effect of PP2 on ionomycin and PMA induced histamine release from human basophils. Our results indicate that PP2 has no significant effect on ionomycin or PMA induced histamine release except at the highest concentration tested (30 μM), (data not shown). PP1 and PP2 alone also failed to either initiate histamine release or modulate spontaneous release at concentrations up to 100 μM (data not shown). In addition, washing the cells after a preincubation with either PP1 or PP2 prior to stimulation had no effect on histamine release suggesting that the effects of both PP1 and PP2 are reversible (data not shown).

We next examined the effects of PP1 and PP2 on the release of LTC_4 and IL-4 from human basophils. As figure 5 demonstrates, 10 μM PP1 and PP2 significantly inhibited anti-IgE induced LTC_4 production. Inhibition was 72±7% and 90±4% in the presence of 10μM PP1 and PP2 respectively (n=5, P=0.04). In contrast, 1 μM PP1 produced a significant enhancement of anti-IgE induced LTC_4 production from 23±12 to 48±15 pmoles per 10^6 basophils (n=5, P=0.04). Similar enhancement effects on LTC_4 production were also observed in the presence of PP2. At 1 μM PP2, anti-IgE induced LTC_4 production from human basophils was enhanced by 123±49% (n=5, P=0.04). As a control, we also investigated the effect of PP1 on fMLP induced LTC_4 production from human basophils. Our results indicate that 10 and 1 μM PP1 have no effect on

fMLP induced LTC_4 production from human basophils (n=2, data not shown). 10 μM PP1 also inhibited anti-IgE induced IL-4 release in a manner that was similar to histamine and LTC_4. At 10 μM PP1 inhibition of IL-4 release was 90±1% (n=2, data not shown). Similar results were also found in the presence of 10 μM PP2 (data not shown).

Discussion

Recent studies have demonstrated that PP1 and PP2 are potent and specific inhibitors of src kinases and have no effect on the Zap-70 family of tyrosine kinases, of which p72syk is a member, and other kinases including Jak 2 and protein kinase A [21, 22]. In our hands, we found that PP1 and PP2 are both potent inhibitors of p53/56lyn phosphorylation following IgE-mediated stimulation of human basophils. Our results also indicate that PP1 and PP2 inhibit other tyrosine phosphorylated proteins but because inhibition of lyn kinase should ablate all downstream events, if the model is correct, it is not possible to know whether PP1 and PP2 inhibit downstream kinases directly. These results are in agreement with studies carried out in the RBL-2H3 cell line. It should be noted, however, that the potency of these drugs in an *in vitro* system is in the nanomolar range but in whole cells concentrations in the micromolar range are required.

The reason for the difference in potency between these two cell systems is unclear, however it may be attributed in part to the permeability of the compound or to the concentration of ATP within the cell. Studies by Hanke *et al* have shown that at certain concentrations of ATP, PP1 becomes competitive with ATP. As a consequence of these results Hanke *et al* predicted that an intracellular concentration of ATP above the millimolar range may act to decrease the potency of PP1 and related compounds [21].

In our hands, we also found that PP1 and PP2 are potent inhibitors of basophil mediator release with an IC_{50} of approximately 3 μM. These results are in agreement with studies carried out in the RBL-2H3 cell line which demonstrated a significant inhibition of mediator release, measured by

the release of β-hexosaminidase, by PP1 at concentrations similar to those found to be effective in the human basophil [22]. No effect was observed on fMLP, ionomycin or PMA induced histamine release in the presence of 10 μM PP1 or PP2 suggesting that these inhibitors are not affecting shared events associated with degranulation. However, PP1 and PP2 at concentrations above 30 μM did have a significant effect on non-IgE mediated histamine release indicating that stimulus dependent selectivity is restricted to concentrations ≤10μM. Indeed, studies have shown that PP1 and PP2 at concentrations above 50-100 μM inhibit other kinases including Zap-70 and Jak 2 [21].

It was interesting that lower concentrations of PP2 (0.3 μM) caused a significant enhancement of histamine release in human basophils, an effect which was also reflected in the release of LTC_4. It is thought in the basophil that histamine release is a balance of activation and desensitization events [30] and that src kinases may play an important role in both of these processes [19, 31]. Indeed, we will be demonstrating in a forthcoming publication that PP1 and PP2 reverse the desensitization of the basophils. Therefore, the observed inhibition and enhancement of histamine release seen in the presence of different concentrations of PP2 may well reflect the inhibitors' ability at lower concentrations to shift the balance of activation and desensitization in such a way that the dominant functional effect is the reversal of desensitization and therefore enhanced secretion.

In conclusion, we have shown that the tyrosine kinase inhibitors PP1 and PP2 are potent inhibitors of anti-IgE induced mediator release from human basophils, possibly through the inhibition of the src kinase, p53/56lyn.

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Figure Legends

Figure 1: The effect of PP1 and PP2 on anti-IgE induced p53/56lyn phosphorylation in human basophils. a) Purified human basophils were preincubated in the presence or absence of either 10 μM PP1 (lane 3) or 10 μM PP2 (lane 4) for 10 minutes at 37° C, and then challenged with 200 ng/ml anti-IgE for 5 minutes. The cells were then lysed in 1% NP-40 lysis buffer. Cell lysates were then incubated with 1μg/ml rabbit anti-human p53/56lyn for 60 minutes at 4° C. The immunoprecipitated proteins were then separated by SDS-PAGE (4-20% gel), transferred to nitrocellulose and analyzed by immunoblotting with anti-phosphotyrosine, 4G10. The tyrosine phosphorylation of p53/56lyn is indicated by an arrow on the right. Lane 1 represents unstimulated basophils, lane 2 human basophils stimulated with anti-IgE and lanes 3 and 4 human basophils stimulated with anti-IgE after a 10 minute preincubation with 10 μM PP1 or 10 μM PP2 respectively. b)Immunoblotting with a mouse anti-human lyn antibody to confirm that the band observed in a) is that of p53/56lyn and to demonstrate equal protein loading. This figure is representative of 3 experiments where the mean purity of the basophils was 92±4% and the mean histamine release was 18±9% after 45 minutes post stimulation.

Figure 2: The effect of PP1 on anti-IgE induced tyrosine phosphorylation in human basophils. Purified human basophils were incubated with various concentrations of PP1 for 10 minutes at 37° C and then challenged with 200 ng/ml anti-IgE for 5 minutes. The reaction was stopped and the proteins solubilised in 1x SDS sample buffer. a) Proteins were then separated by SDS-PAGE (4-20% gels), transferred to nitrocellulose and analyzed by immunoblotting with anti-phosphotyrosine, 4G10. The arrows indicate the mass extrapolated from markers (left hand side) and the location of the various tyrosine phosphorylated proteins (right hand side). b)Immunoblotting with an anti-syk antibody to demonstrate equal protein loading. This figure is representative of 3 experiments where the mean purity of the basophils was 92±1% and the mean histamine release was 24±3% after 45 minutes post stimulation.

Figure 3: The effect of PP1 on histamine release induced from human basophils by various stimuli. A) Human basophils were incubated with various concentrations of PP1 for 10 minutes at 37 °C and then challenged with either 200 ng/ml anti-IgE (●) or 1 μM fMLP (○). Mediator release was allowed to proceed for 45 minutes, the supernatant recovered, and histamine release measured. Control histamine release was 50±12% for cells challenged with anti-IgE (n≥4 for all points) and 78±13% for cells challenged with fMLP (n≥5 for all points). Results are expressed as mean±sem and * indicates that the data point reached statistical significance.

Figure 4: The effect of PP2 on histamine release induced from human basophils by various stimuli. A) Human basophils were incubated with various concentrations of PP2 for 10 minutes at 37 °C and then challenged with either 200ng/ml anti-IgE (●) or 1 μM fMLP (○). Mediator release was allowed to proceed for 45 minutes, the supernatant recovered, and histamine release measured. Control histamine release was 54±6% for cells challenged with anti-IgE (n≥9 for all points) and 76±9% for cells challenged with fMLP (n=5 for all points). Results are expressed as mean±sem and * indicates that the data point reached statistical significance.

Figure 5: The effect of PP1 and PP2 on anti-IgE induced LTC_4 production from human basophils. Human basophils were incubated with various concentration of either PP1 (●) or PP2 (○) for 10 minutes at 37° C and then challenged with 200 ng/ml anti-IgE. Mediator release was then allowed to proceed for 45 minutes, the supernatant recovered, and LTC_4 production measured. Control LTC_4 production was similar for both sets of experiments at approximately 23±12 pmoles/106 basophils. Results are expressed as mean±sem and * indicates that the data point reached statistical significance.

Figure 1

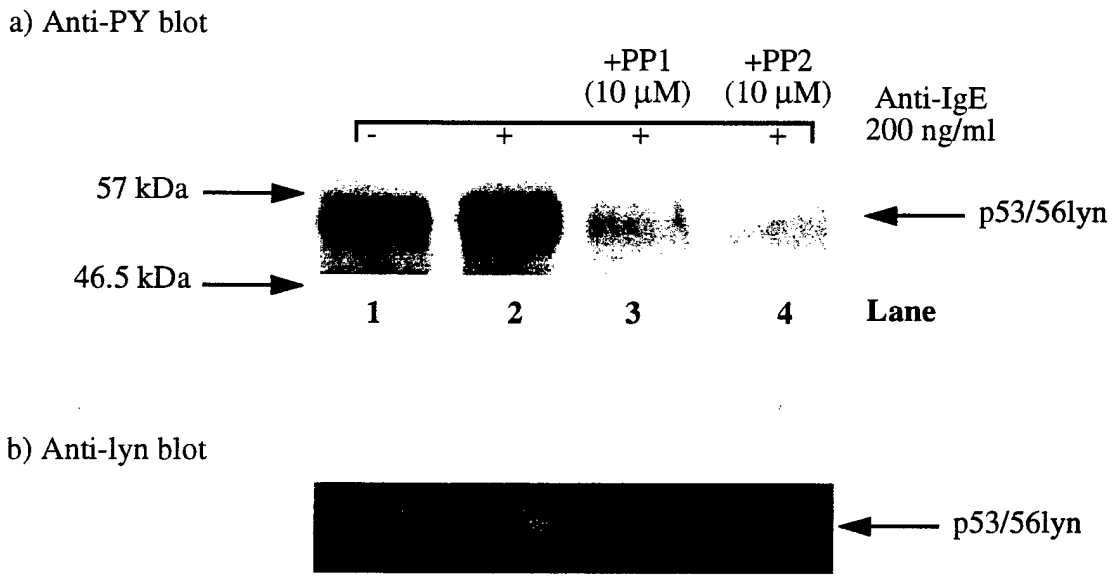
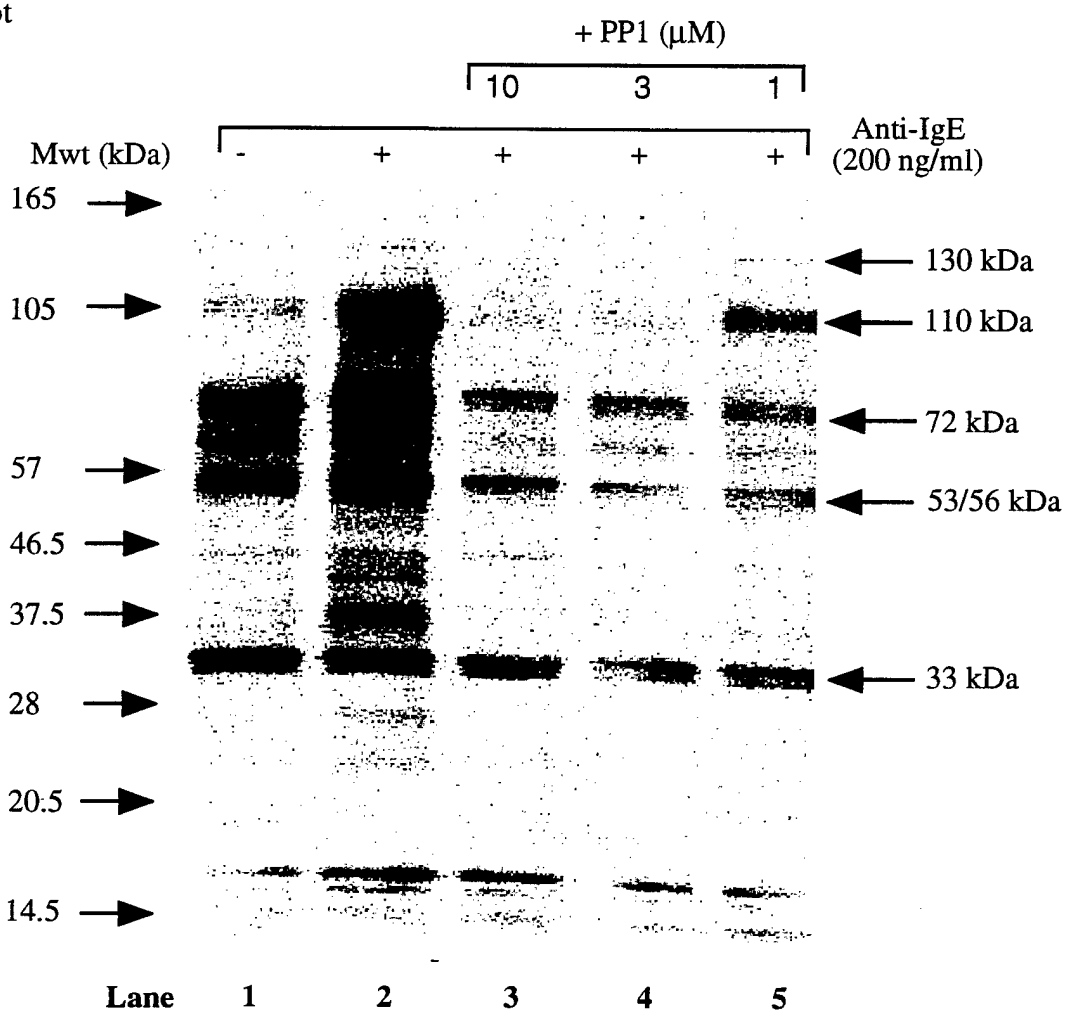

a) Anti-PY blot
+PP1
(10 μM)
+PP2
(10 μM)
Anti-IgE
200 ng/ml
-
+
+
+
57 kDa
46.5 kDa
p53/56lyn
1
2
3
4
Lane
b) Anti-lyn blot
p53/56lyn

Figure 2

a) anti-PY blot



b) anti-syk blot

Figure 3

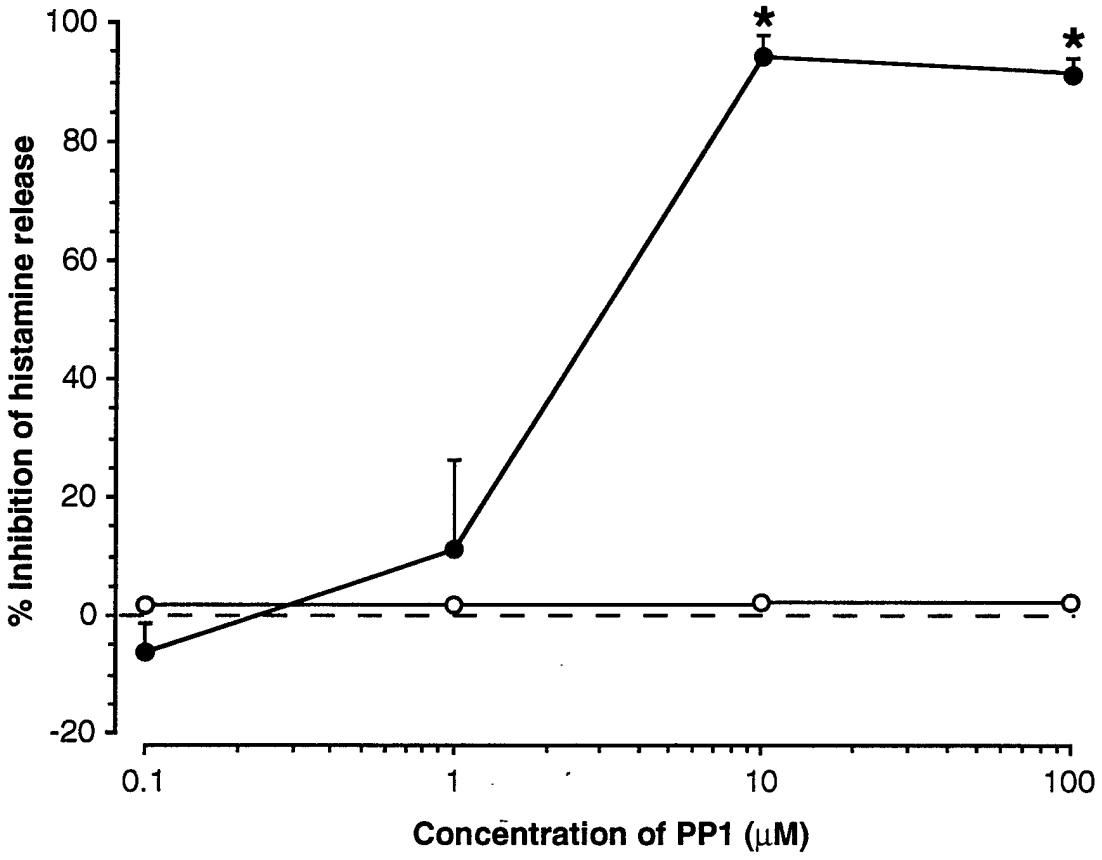

100
80
60
40
20
0
-20
% Inhibition of histamine release
*
*
0.1
1
10
100
Concentration of PP1 (μM)

Figure 4

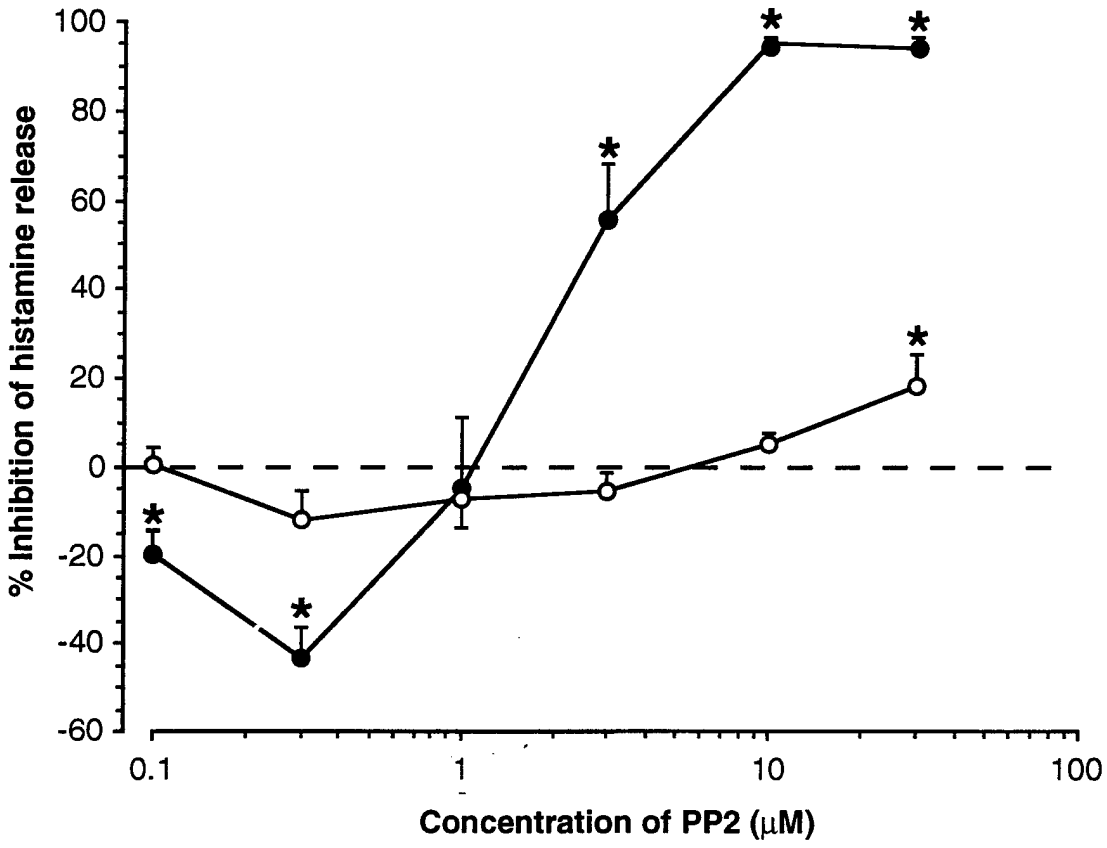
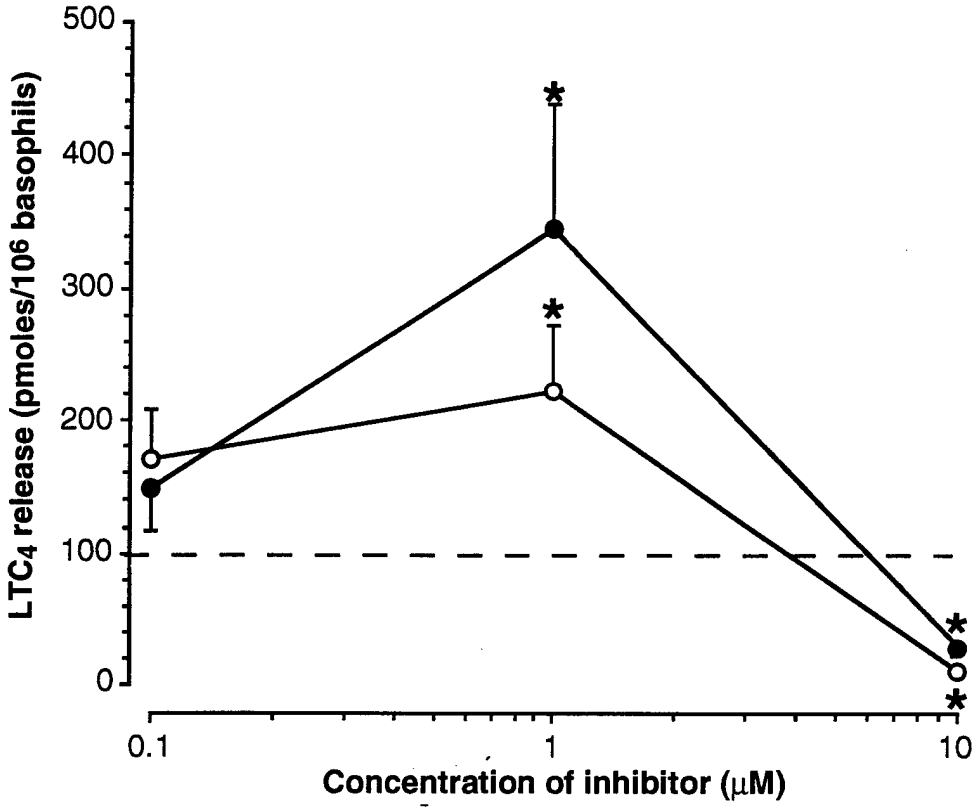

100
80
60
40
20
0
-20
-40
-60
% Inhibition of histamine release
0.1
1
10
100
Concentration of PP2 (μM)
*
*
*
*
*
*

Figure 5


500
400
300
200
100
0
LTC4 release (pmoles/10^6 basophils)
0.1
1
10
Concentration of inhibitor (μM)
*
*
*
*

3432

THE ROLE OF P53/56LYN AND P72SYK IN IGE-MEDIATED SIGNAL TRANSDUCTION IN HUMAN BASOPHILS. SE Lavens-Phillips and DW MacGlashan, Jr. JHAAC. Baltimore, MD 21224.

The mechanism(s) which lead from the crosslinking of IgE on the surface of human basophils, to the release of histamine have not been completely elucidated. However, there is evidence in the rodent mast cell and associated cell lines suggesting a role for tyrosine kinases in IgE-mediated signalling. We have demonstrated in human basophils an increase in tyrosine phosphorylation of several proteins in the 130, 110, 75-65, 53/56, 33 and 26kDa range. In general, tyrosine phosphorylation began as early as 30 seconds after stimulation and in the majority of cases reached a plateau within 1 to 3 minutes. Tyrosine phosphorylation was then usually maintained at this level for 15 minutes post stimulation even in the absence of extracellular calcium. However, for some tyrosine phosphorylated proteins the kinetics was somewhat slower, with phosphorylation beginning within 1 minute after stimulation and continuing to increase for up to 15 minutes post stimulation. This was especially prominent in the proteins tyrosine phosphorylated at 130, 110, and 26kDa. Immunoprecipitation with anti-lyn or syk followed by western blotting with anti-phosphotyrosine showed marked stimulus dependent phosphorylation of the 53/56 and 70kDa bands respectively. Activation of human basophils by other stimuli including fMLP and two non-receptor mediated stimuli, ionomycin and PMA, failed to increase the phosphorylation state of either the 53/56 or 70kDa bands, confirming activation of p53/56lyn and p72syk is restricted to stimulation through the FceRI. In conclusion, we have shown that the tyrosine phosphorylation of both p53/56lyn and p72syk is an early event in the IgE-mediated signalling cascade.

259.25

Pharmacology of Human Basophil (HB) Desensitization

S.E. Lavens-Phillips and D.W. MacGlashan. Jr. JHU-AAC Baltimore. MD 21224

Mediator release from HB represents a balance of activating and down-regulating signal transduction cascades. Operationally, down-regulation/desensitization (DESN) can be observed by stimulating HB with antigen or anti-IgE antibody in the absence of extracellular calcium (Ca) and testing for a change in function by adding Ca back to the reaction. The longer one waits before adding Ca back. the less responsive the cells become. We have examined the effects of kinase inhibitors on DESN. To do these experiments. it must be possible to wash the drug from the cells before adding Ca back to the reaction in order to assess only the effects of the drug on the DESN phase. It has been proposed that DESN is mediated by the action of protein kinase C so we examined the effects of relatvively selective PKC inhibitors of the indolylmaleimide class on DESN. Brief washing effectively removes Bis II and Ro-31-8220 from HB. DESN in the presence of 0.5 to 2 μM Bis II or Ro-31-8220 is unchanged compared to HB desensitized in the absence of the drugs. A variety of positive controls demonstrate that these concentrations effectively inhibit functional PKC activity in HB. These experiments suggest that PKC does not mediate DESN so we next questioned whether early tyrosine kinase (TK) activity was necessary. We and others have found that Go-6976 is a potent inhibitor of early TK at 50-100 nM. This drug can also be effectively washed from HB (as above). DESN in the presence or absence of 100 nM Go-6976 is equivalent. The results suggest that not only is PKC activity not necessary for DESN but also suggest that activation of early TKs is not necessary.

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IgE Interacts with FcεRI to Induce FcεRI Up-reglation on Human Basophils (HB). DW MacGlashan, Jr, J McKenzie, SE Lavens-Phillips, AJ Henry, BJ Sutton, HJ Gould Johns Hopkins-AAC, Baltimore MD 21224 and Kings College London, UK WC2B 5RL.

FcεRI expression on HB is altered by the presence or absence of IgE Ab. It is not clear whether this alteration in cell surface expression is coordinated by an interaction between IgE Ab and FcεRI itself or through another IgE binding protein on HB. Four possibilities were examined, IgE interacting through CD32 (IgG receptor on HB), CD23, εBP or FcεRI. HB (40-99%) were cultured for 7 days (IMDM media/10 ng/ml IL-3) ±IgE (1μg/ml), which caused a 3-10 fold up-regulation of FcεRI. FcεRI expression was detected by flow cytometry using the monoclonal anti-FcεRIα Ab, 22E7. Previous studies excluded a role for CD32 as IgG did not cause or influence IgE-induced FcεRI expression. α-Lactose had no influence on the ability of IgE to up-regulate FcεRI, excluding a role for εBP. A role for CD23 was excluded by 4 different studies. Purified HB were examined by flow cytometry for CD23 expression; no difference between MHM6 (anti-CD23) and isotype control IgG was found. Purified HB were also not found to express mRNA for CD23 as determined by RT-PCR while the same PCR primers could detect mRNA in lymphocytes (32 cycles). Anti-CD23 (MHM6) was not found to induce or alter IgE-induced up-regulation of FcεRI. Finally, two recombinant IgE-Fc's, one wild type (IgE-Fc(WT)), one mutant (IgE-Fc(R334S)), were used to up-regulate FcεRI. The IgE-Fc(WT)and IgE-Fc(R334S) have been previously shown to have equivalent binding characteristics to CD23 but IgE-Fc(R334S) has a 33 fold lower affinity for binding to FcεRI. When these fragments were used to up-regulate FcεRI on HB, IgE-Fc(WT) was found to up-regulate expression with 30 fold greater potency than IgE-Fc(R334S) (EC50 of 1.7×10^{-10} M vs 4.9×10^{-9} M for IgE-Fc(WT) and IgE-Fc(R334S), respectively). Collectively these data indicate no role for CD23 and indicate that IgE interacts with FcεRI to induce its up-regulation.

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